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Transcriptomics hit the target: Monitoring of ligand-activated and stress response pathways for chemical testing

Alice Limonciel [a,*], Konrad Moenks [b], Sven Stanzel [c], Germaine L. Truisi [d,e], Céline Parmentier [f], Lydia Aschauer [a], Anja Wilmes [a], Lysiane Richert [f,h], Philip Hewitt [d], Stefan O. Mueller [d,e], Arno Lukas [b], Annette Kopp-Schneider [c], Martin O. Leonard [g], Paul Jennings [a]

[a] Department of Physiology, Medical University of Innsbruck, Innsbruck, Austria
[b] Emergentec Biodevelopment GmbH, Vienna, Austria
[c] Division of Biostatistics (C060) Medical Biostatistics, German Cancer Research Center (DKFZ), Heidelberg, Germany
[d] Non-Clinical Safety, Merck Serono, Merck KGaA, Darmstadt, Germany
[e] Institut für Angewandte Biowissenschaften, Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany
[f] KaLy-Cell, Plobsheim, France
[g] Centre for Radiation, Chemical and Environmental Hazards, Public Health England, Chilton, Didcot OX11 0RQ, UK
[h] EA4267, Université de Franche-Comté, Besançon, France

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ABSTRACT

High content omic methods provide a deep insight into cellular events occurring upon chemical exposure of a cell population or tissue. However, this improvement in analytic precision is not yet matched by a thorough understanding of molecular mechanisms that would allow an optimal interpretation of these biological changes. For transcriptomics (TCX), one type of molecular effects that can be assessed already is the modulation of the transcriptional activity of a transcription factor (TF). As more ChIP-seq datasets reporting genes specifically bound by a TF become publicly available for mining, the generation of target gene lists of TFs of toxicological relevance becomes possible, based on actual protein-DNA interaction and modulation of gene expression. In this study, we generated target gene signatures for Nrf2, ATF4, XBP1, p53, HIF1a, AhR and PPAR gamma and tracked TF modulation in a large collection of *in vitro* TCX datasets from renal and hepatic cell models exposed to clinical nephro- and hepato-toxins. The result is a global monitoring of TF modulation with great promise as a mechanistically based tool for chemical hazard identification.

1. Introduction

Risk assessment of chemicals *in vitro* is becoming more and more accepted as a foreseeable replacement for *in vivo*-based strategies (Jennings, 2014). As in animal-based tests, it is the combination of hazard and exposure measurements that allows to assess the risk related to the use of a given chemical. Thus *in vitro*, both a rigorous monitoring of compound uptake and intra-cellular concentration and methods to identify chemical hazard are required. While cell viability endpoints are well established, alone they are not sufficient for chemical hazard characterisation since they provide little mechanistic information. As is the case for *in vivo* testing, survival as a sole indicator is not enough for toxicity assessment *in vitro*, especially for chronic toxicity testing where a non-lethal concentration of chemical may still interfere with the system's physiology. At the organism level, this could be the onset of long-term effects such as cancer and other progressive diseases

Abbreviations: AD, adefovir dipivoxil; AhR, aryl hydrocarbon receptor; AMIO, amiodarone; APAP, acetaminophen; ATF4, activating transcription factor 4; CAA, chloroacetaldehyde; $CdCl_2$, cadmium chloride; ChIP-seq, chromatin immunoprecipitation followed by DNA sequencing; CID, cidofovir; CLOD, clodronate; CP, cisplatin; CPZ, chlorpromazine; CsA, cyclosporine A; EMD, EMD335823; FENO, fenofibrate; HIF1a, hypoxia inducible factor 1, alpha subunit; HYPO, hypoxia (1% O_2); IBU, ibuprofen; IF, ifosfamide; MET, metformin; Nrf2, nuclear factor (erythroid-derived 2)-like 2 (aka Nfe2l2); p53, tumour protein 53 (aka tp53); PHH, primary human hepatocyte; PPARG, peroxisome proliferation-activated receptor gamma; PRH, primary rat hepatocyte; ROS, reactive oxygen species; ROSI, rosiglitazone; RPTEC/TERT1, human renal proximal tubule cell line transfected with human telomerase; TCX, transcriptomics; TF, transcription factor; TROG, troglitazone; VALP, valproic acid; XBP1, X-box binding protein 1; ZOL, zoledronate.

* Corresponding author at: Department of Physiology and Medical Physics, Medical University of Innsbruck, Fritz-Pregl-Strasse 3/1, 6020 Innsbruck, Austria. Tel.: +43 512 9003 70839.

E-mail address: alice.limonciel@i-med.ac.at (A. Limonciel).

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that do not originate from cell death, but rather from disruptions in normal cellular functions and interference with the defence pathways that keep them in check.

In vitro toxicity testing is moving towards the integration of classical cell viability endpoints with measures of cellular functions into batteries of stress and toxicity endpoints. Such functional endpoints monitor changes in energy metabolism (e.g. glycolysis rates (Limonciel et al., 2011)), cytochrome P450 activities, specific transporter activities (Aschauer et al., 2014), epithelial monolayer barrier function (trans-epithelial electrical resistance (TEER) (Wilmes et al., 2014a)) and electrical activity of neurons (Schultz et al., 2014). These functions contribute to organ/tissue function, but also to cellular homeostasis, and on a larger scale to whole-body homeostasis. A number of evolutionarily conserved stress response pathways constitute a first level of protection against the deregulation of these cellular assets (Jennings, 2013). For instance, the response activated by the transcription factor (TF) Nrf2 (nuclear factor (erythroid-derived 2)-like 2, aka Nfe2l2) is highly conserved in many organisms, inducible in numerous cell types and represents an essential protective mechanism against increased levels of reactive oxygen species (ROS), which are a common underlying factor in xenobiotic-induced injury, ischemia–reperfusion injury, cardiotoxicity, diabetes and aging (Baird and Dinkova-Kostova, 2011). The Nrf2 protein is constitutively expressed, sequestered in the cytosol and largely targeted for proteasomal degradation under homeostatic conditions. When the levels of alkylating or oxidative species (including ROS) increase in the cytosol, Nrf2 degradation is halted, and the protein translocates to the nucleus to modulate the expression of a number of genes that will form a coordinated response to the ongoing attack to the cell. A number of other TFs act similarly to protect most mammalian cells against ER stress (activating transcription factor 4 (ATF4), X-box binding protein 1 (XBP1) (Kim et al., 2008)), DNA damage (tumour protein p53 (Reinhardt and Schumacher, 2012)), hypoxia (hypoxia inducible factor 1, alpha subunit (HIF1a) (Semenza and Wang, 1992)) and more. While these TFs trigger the primary response to a specific type of ongoing stress, their effect on the expression of their target genes is almost immediately measurable. This prompt transcriptomic response to stress is also observed upon binding of ligands to nuclear receptors, another family of TFs including the aryl hydrocarbon receptor (AhR) and peroxisome proliferation-activated receptors (PPARs), which respond to ligand binding notably by modulating the transcription of genes involved in the metabolism of their ligand (Denison et al., 2011; Varga et al., 2011). Thus, whether it is for a TF in charge of a stress response or a ligand-activated nuclear receptor, transcriptomic data (TCX) represents an ideal playground to investigate TF modulation with an appropriate list of target genes (Jennings et al., 2013).

In order to explore the influence of chemicals on specific TF-activated pathways, we first mined the literature for the most accurate datasets demonstrating TF-specific target gene activation. To this end, we focused primarily on studies that combined chromatin immunoprecipitation followed by DNA sequencing (ChIP-seq) with gene expression datasets (microarray or mRNA-seq) to generate TF target gene lists from independent studies. The resulting gene lists were used to study TF modulation in the TCX data produced in the Predict-IV project on three state-of-the-art cell models: the telomerase transfected human proximal tubule cell line RPTEC/TERT1, and primary human and rat hepatocytes (PHH and PRH, respectively). With this novel approach, we could monitor time-dependent modulations of TFs, identify differential patterns of activation in the three cell models within each TF signature and conclude on the promising application of such a strategy for hazard identification.

2. Methods

2.1. Bait gene lists

Gene lists were generated from previously published ChIP-seq studies where the TF of interest was activated a few hours prior to cell lysis. Typically, proteins and DNA were cross-linked, chromatin was immuno-precipitated with a TF-specific antibody and genes that were identified in the TF-bound chromatin fragments by sequencing and differentially expressed constituted the gene list. Table 1 summarises the experimental conditions used in the reference publications for activation of the TFs, DNA binding analysis and gene expression analysis. Entrez Gene IDs were used as the common identifier across this study, thus gene IDs were converted from the original publications to human and rat IDs using BioMart version 0.7 (Kasprzyk, 2011). The lists were then manually curated to update gene names according to the HGNC nomenclature. All Entrez Gene IDs and full gene names are reported in Supplementary Table 1.

2.2. Cell culture

Detailed methodologies for cell culture, toxin exposure and transcriptomics have been published in accompanying papers of this special issue (Bellwon et al., 2014; Wilmes et al., 2014b). In brief, RPTEC/TERT1 cells were cultured on 0.2 μm aluminium oxide filter inserts in hormonally-defined medium and allowed to mature into a functional monolayer for 10 days after confluence before compound exposure started (Aschauer et al., 2013; Wieser et al., 2008). Primary human hepatocytes (PHH) from three donors were isolated using the two-step perfusion technique described by Lecluyse and Alexandre (2010) and seeded and cultured in sandwich culture as described by Parmentier et al. (2013). Treatment of PHH was started at day 2 of culture. Primary rat hepatocytes (PRH) were isolated from male Wistar rats according to Seglen (1976) using a two-step liver perfusion method. The cells were cultured in a so-called "sandwich configuration" using collagen type I as described by Tuschl et al. (2009). Treatment of PRH was started on day 3 after seeding.

For all cell models, a repeat-dose treatment regime was applied with renewal of medium and compound every 24 h. RNA was isolated using the RNeasy Mini Kit (QIAGEN) for transcriptomic analysis after 1, 3 and 14 days of exposure. Since the Predict-IV project focused on the study of pharmaceutical active principals, the test compounds could be chosen based on information available from the clinics on their known target organs of toxicity in humans. In RPTEC/TERT1 cells, hypoxia (HYPO, 1% O_2) and nine nephrotoxins were tested: eight clinical compounds (adefovir dipivoxil (AD), chloroacetaldehyde (CAA), cyclosporine A (CsA), cidofovir (CID), cisplatin (CP), clodronate (CLOD), ifosfamide (IF) and zoledronate (ZOL)) and the environmental contaminant cadmium chloride ($CdCl_2$). In PHH and PRH, eleven clinical hepatotoxins were tested: acetaminophen (APAP), amiodarone (AMIO), chlorpromazine (CPZ), CsA, EMD335823 (EMD), fenofibrate (FENO), ibuprofen (IBU), metformin (MET), rosiglitazone (ROSI), troglitazone (TROG) and valproic acid (VALP). For each compound, a low and high concentration were selected based on preparatory experiments and the high concentration was chosen to induce no more than 10% cytotoxicity, according to intracellular ATP levels (data not shown). Thus, for the two hepatic models, even when the applied concentrations were different, the objective was to induce similar levels of stress in order to allow a better comparison of the effects between the two models. Detail of treatments is given in Supplementary Table 2. All conditions were tested in triplicate.

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