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Assessing Hepatotoxic and Nephrotoxic Compounds in HepaRG and RPTEC Cells by Key Signaling Events Using Multiplexed Microsphere-Based Sandwich Immunoassays

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INTRODUCTION

The implementation of new approach methods (NAM) to reduce, replace or refine animal testing is a major challenge in risk assessment. One aspect that has to be considered is their applicability in different regulatory domains, such as pesticides or contaminants. In addition, the robustness and the accuracy of testing has to be evaluated.

OBJECTIVES

In this study, the effects of substances from different regulatory domains, that are showing different forms of hepatotoxicity or nephrotoxicity in vivo, were assessed with the HepaRG and RPTEC/tert cell models by analysis of eight cellular key processes: Translation, protein degradation, meiosis/ mitosis, thermal stress, transcription apoptosis, autophagy, and hypoxic stress.

Cyproconazole
- fungicide
- *in vivo:* hypertrophy, steatosis

Fluxapyroxad
- fungicide
- *in vivo:* necrosis, steatosis

Azoxystrobin
- fungicide
- *in vivo:* hepatocellular cell death, hypertrophy

Chlorotoluron
- herbicide
- *in vivo:* tumors, tubular hyperplasia

Thiabendazole
- fungicide
- *in vivo:* lesions of the biliary epithelium, pelvis and tubular hyperplasia

Phenylphenole
- fungicide
- *in vivo:* oxidative stress, cell death

PFOS
- anthropogenic fluorosurfactant, PBT
- *in vivo:* steatosis, cholestasis

PFOA
- anthropogenic fluorosurfactant, PBT
- *in vivo:* steatosis, cholestasis

Aflatoxin B1
- mycotoxin
- *in vivo:* tumors

Cadmium ($CdCl_2$)
- heavy metal
- *in vivo:* tumors

Lasiocarpine
- pyrrolizidine alkaloid
- *in vivo:* veno-occlusive disease, tumors

Figure 1: Test substances, pesticides (red underlay) and food contaminants (blue underlay), used in cell culture experiments to identify or confirm toxicological mode of action.

Table 1: Proteins and modification of proteins indicative of certain cellular endpoints assessed by 8-plex microsphere-based sandwich immunoassays.

Cell Process	Protein / protein modification
Translation	phospho eIF4b
Protein Degradation	Lys48 ubiquitinylation
Meiosis/ Mitosis	phospho Histone H3
Thermal stress	HSP70
Transcription	phospho RNA Pol II
Apoptosis	cleaved PARP
Autophagy	LC3B
Hypoxic stress	HIF 1 alpha

RESULTS

The analysis revealed that individual xenobiotics triggered various cell processes, reflecting expected effects as well as providing novel insights for so-far unknown modes of action.

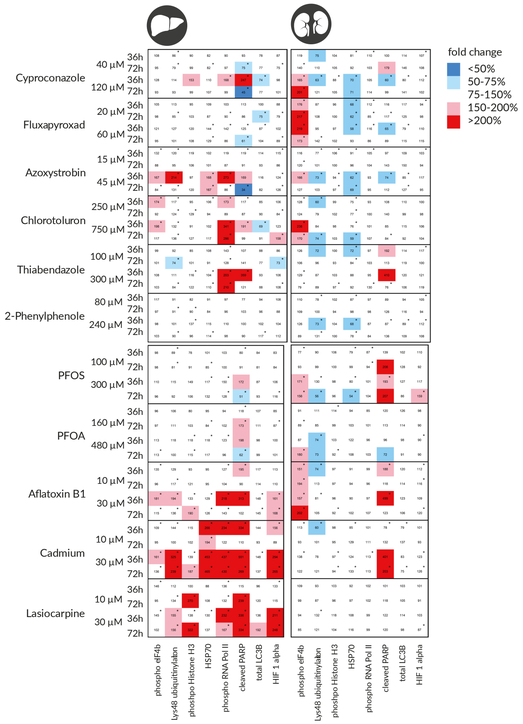

Figure 2: Effects on protein expression or protein modification of selected markers observed in HepaRG and RPTEC after 36 h and 72 h of incubation with the test substances using 8-plex microsphere-based sandwich immunoassays. Results are shown as mean of 3 independent experiments measured in technical duplicates, normalized to solvent controls. Statistical differences to the solvent control were calculated with bootstrapping (*p<0.05).

SUMMARY AND OUTLOOK

- Results indicate that the combination of the cell models with the novel multiplexed sandwich immunoassay can be applied for the toxicological evaluation of new substances without the use of animals.
- Future experiments will cover dose-response curves and the application of mixtures of chemicals.

Method

We developed an 8-plex microsphere-based sandwich immunoassay for the analysis of cellular key functions (Table 1 & Figure 3) on a bead-based array system to investigate toxicological effects on cell functions. Here fluorescently-coded beads (Bead-ID fluorescence) are used to run sandwich immunoassays in multiplex fashion. Capture antibodies are used to enrich analytes of interest e.g. phoshorylated Histone H3. In a second step biotinylated detection antibodies bind to their respective analyte. The antibody-protein sandwich complexes are finally labeled with phycoerythrin-streptavidin conjugate (reporter fluorescence). The assay is read out on a flow system detecting reporter and bead ID fluorescence. Typically 5 µg - 20 µg protein extract is sufficient to relatively quantify 10 - 20 proteins and/ or modifications.

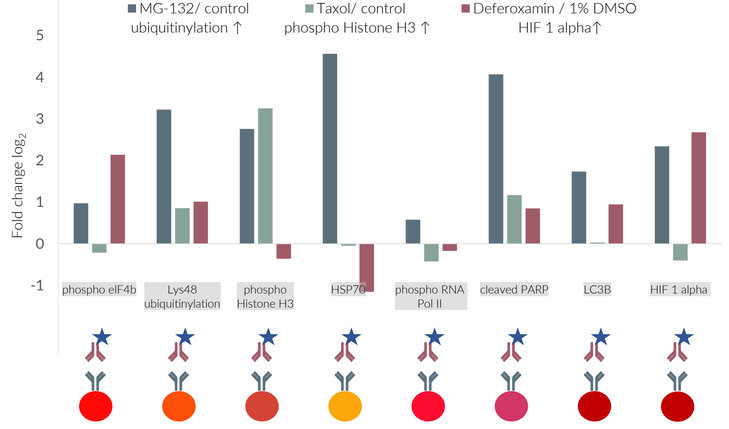

Figure 4: 8-plex microsphere-based sandwich immunoassays for the analysis of cellular key functions. HEPG2 cell cultures were treated with three compounds to trigger respective responses in cell functions: MG-132 for proteasome inhibition (ubiquitinylation ↑), Taxol for cell cycle arrest (phospho Histone H3 ↑), and Deferoxamin for hypoxia (HIF 1 alpha ↑). Eight different color-coded beads (bead ID fluorescence) conjugated with respective capture antibodies were incubated with the protein extracts overnight. The beads were then removed from the sample using a magnetic bead handling robot. The detection step was carried out by incubating the solution with respective biotinylated detection antibodies, followed by a 3rd incubation step with a phycoerythrin-strepatvidin conjugate (reporter fluorescence). Bead ID fluorescence and reporter fluorescence were read out on FLEXMAP 3D (Diasorin, LUMINEX Corp, Austin TX). The figure shows the median fluorescence intensities normalized to the respective solvent control.

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