

Cytotoxicity studies on 3D primary liver microtissues.

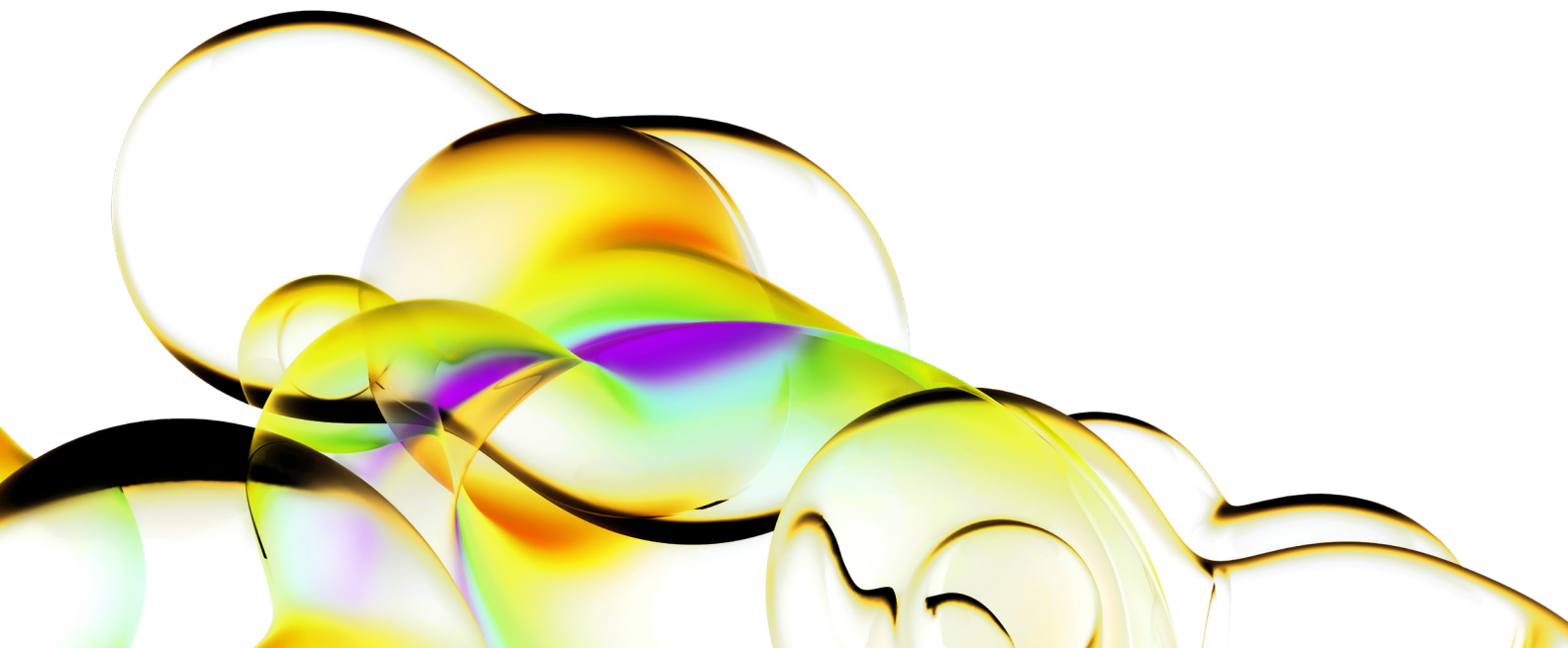
Key features

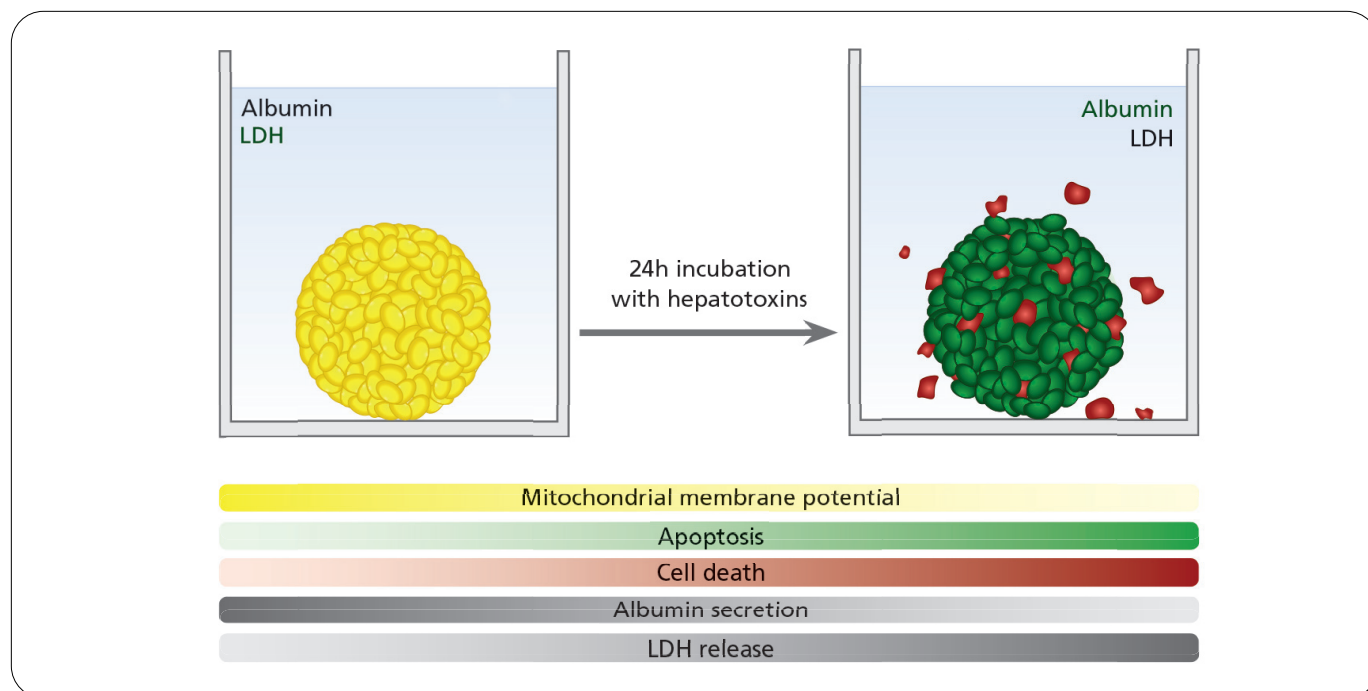
- Primary liver microtissues as hepatotoxicity 3D model
- Complementary readouts using high-content imaging and biochemical assay detection technologies
- High quality imaging of 3D microtissues using high-content imaging

Background

Liver toxicity remains one of the main reasons for drug failure in clinical trials. Improving preclinical toxicity testing is a major prerequisite for improving the drug de-risking process. Early compound de-risking decisions require *in vitro* data with the highest possible biological relevance. Three dimensional (3D) microtissues, or spheroids, are one of the most well characterized models for 3D culture and cell-based drug screening due to their reproducibility and similarity to the *in vivo* situation [Breslin and O'Driscoll, 2012]. Liver microtissues generated via the co-culture of primary human hepatocytes and non-parenchymal cells are used to better mimic the complex structure of human liver [Godoy et al., 2013].

Here, we present a proof of concept study to demonstrate the feasibility of a 4-color toxicity assay using living Troglitazone and Staurosporine treated primary human liver microtissues. In this assay, the combination of high-content imaging with biochemical assay detection provides complementary readouts for assessing phenotypic changes and potential toxicological pathways beyond general cytotoxicity markers.





Application

Imaging and biochemical assays were performed as a complementary approach for studying liver toxicity using a microtissue 3D model (Figure 1). 3D InSight™ Human Liver Microtissues consisting of primary human hepatocytes co-cultured with non-parenchymal Kupffer cells were produced using the GravityPLUS™ system from InSphero AG. This system allows for automation of the hanging drop method and provides an optimal tool for generating microtissues amenable to cell-based drug screening [Drewitz et al., 2011]. Once grown, the microtissues were transferred to 384-well LoBase SensoPlate™ Plus (Greiner, 781865) plates. Prior to this step, the plates were coated to reduce cell attachment. Microtissues were treated with the hepatotoxins Troglitazone and Staurosporine, incubated in a total volume of 50 µL medium for 24 h at 37 °C and 5% CO₂. Automated liquid handling was done using the JANUS® Automated Workstation fully integrated with a plate::handler™ workstation.

Biochemical assays: After compound treatment, 35 µL of the supernatant was transferred to a 384-well StorPlate™ (Revvity, 6008590) from each well. 25 µL of the supernatant, plus 25 µL reaction solution from the LDH Cytotoxicity Assay Kit (Cayman, 10008882), was then transferred from the StorPlate to a 384-well ViewPlate™ (Revvity, 6007470) and incubated according to the assay kit instructions.

Absorbance was measured at 490 nm on a EnSpire® multimode plate reader. The StorPlate with the remaining supernatant was sealed and stored at 4 °C overnight. The next day, 5 µL of the supernatant was transferred to a 384-well AlphaPlate™ (Revvity, 6005350). The amount of albumin in the supernatant was determined using the AlphaLISA® Albumin kit (Revvity, AL294C), according to the instructions. The AlphaLISA signal was detected using the EnSpire plate reader.

Imaging assays: After compound treatment, microtissues were stained for 45 min with staining solution in medium containing 15 µg/mL Wheat Germ Agglutinin (WGA) Alexa Fluor® 350 Conjugate (Life Technologies, W11263), 5 µM CellEvent™ (Life Technologies, C10423), 300 nM TMRM (Life Technologies, T-668) and 50 nM SYTOX® Red (Life Technologies, S34859). Imaging was performed on the Operetta high-content analysis system using a 20x high NA objective (60 µM stack, 10 µM plane distance, confocal mode). The maximum intensity projection images were analyzed using Harmony® high-content imaging and analysis software.

The WGA channel was used for tissue segmentation. In the tissue region, the mean intensities of TMRM, CellEvent™ and SYTOX® stain were measured.

Additionally, isolated SYTOX® positive single cells were counted. Using this analysis sequence, the dose-dependent alterations in mitochondrial membrane potential, apoptosis, cell death and microtissue degradation were quantified in order to describe typical phenotypic changes upon treatment with hepatotoxins (Figure 2). Staurosporine and Troglitazone gradually damaged the mitochondrial membrane potential and induced apoptosis,

reflected by a successive decrease in TMRM intensity, as well as an increase in CellEvent™ intensity. High compound concentrations (20 µM Staurosporine, 100 µM Troglitazone) led to a strong increase in cell death in microtissues, and detachment of dead cells from the microtissues. Staurosporine treatments higher than 50 µM caused disaggregation of the entire microtissues.

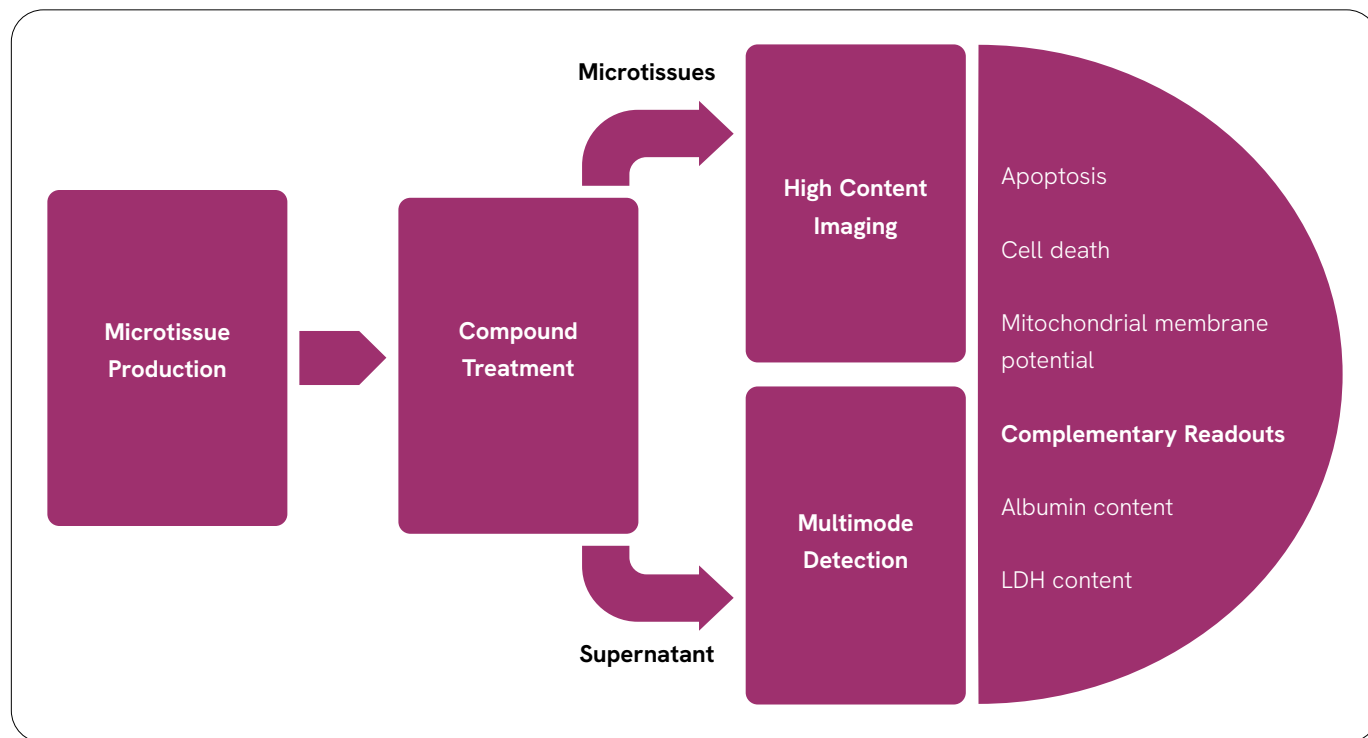


Figure 1: Flow chart of the hepatotoxicity assay procedure. Liver microtissues were produced using the hanging drop method. After compound treatment for 24 h, both the microtissues and their supernatants were analyzed on the Operetta high-content analysis system and the plate reader, respectively. High-content imaging and multimode detection technologies enabled the characterization of hepatotoxicity with complementary readouts.

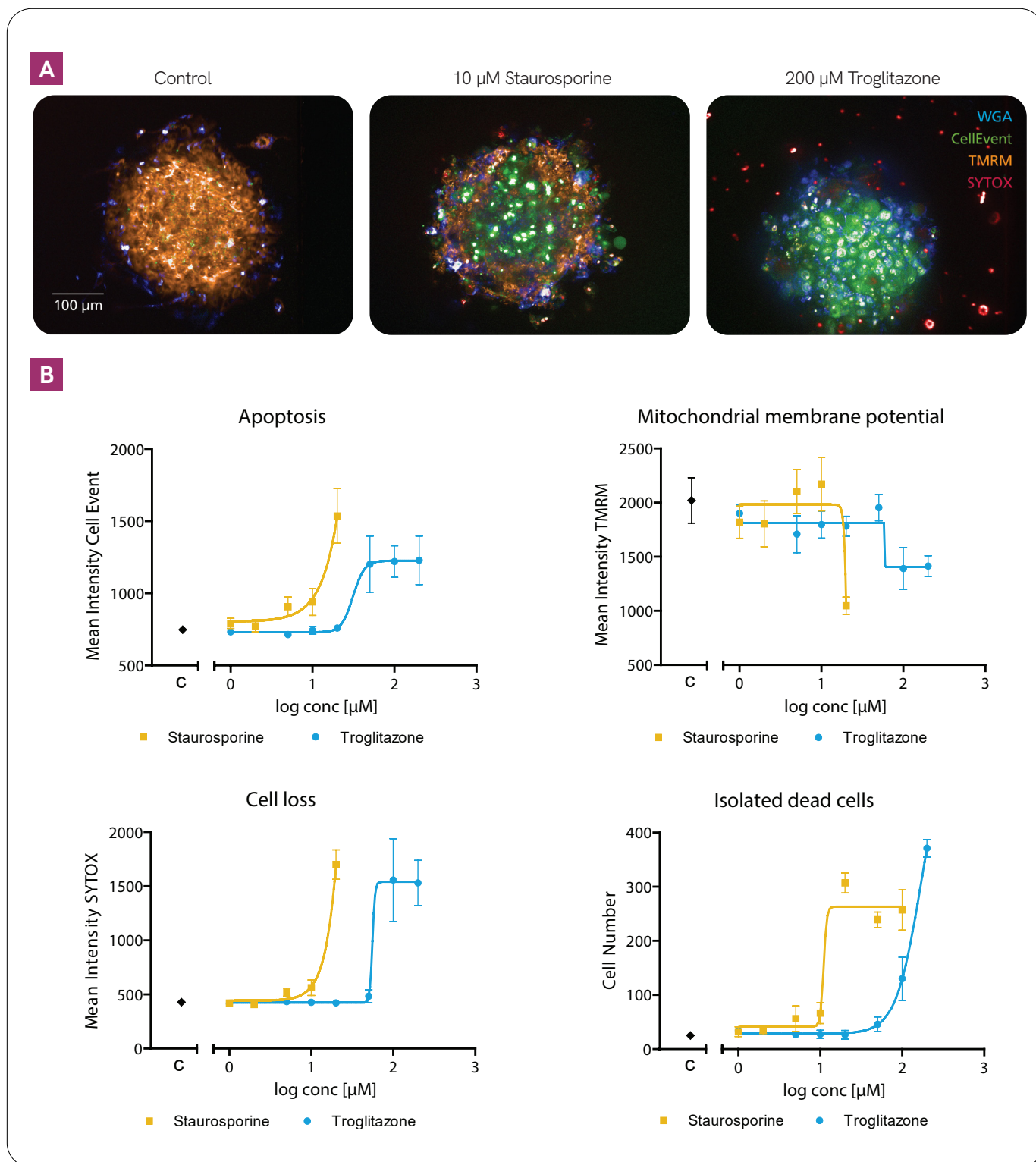


Figure 2: Images and evaluation of high content imaging assay. (A) High-content images (20x high NA, maximum intensity projection of six planes, stack 0-60 µm, confocal mode) of a non-treated control microtissue (left), a Staurosporine-treated microtissue (middle) and a Troglitazone-treated microtissue (right). After 24 h treatment, all tissues were stained with WGA (blue), CellEvent™ (green), TMRM (yellow) and SYTOX® (red). The compound treatment caused a breakdown of the mitochondrial membrane potential and induced apoptosis, resulting in decreased TMRM intensity and increased CellEvent™ intensity compared to the control tissue. Increasing compound concentrations gradually led to cell death in the microtissue and the detachment of single cells from microtissues as shown by an increase in the amount of the dead cell stain, SYTOX®. (B) Quantification of hepatotoxicity in human liver microtissues: Dose-response curves for Troglitazone and Staurosporine deduced from four readouts reflecting major phenotypic changes (n=3 wells).

To detect toxicity in primary liver microtissues, the levels of two common toxicity markers, Lactate Dehydrogenase (LDH) and Albumin, were also determined in the supernatant of the tissues. Elevated LDH levels are an indicator for liver damage, whereas Albumin is continuously secreted by healthy liver cells and the secretion is decreased under toxic conditions. Treatment of the microtissues with Staurosporine resulted in a dose-dependent increase in LDH, but had only a slight impact on the Albumin content in the supernatant. In contrast, Troglitazone treatment led to a decrease in Albumin content, but had almost no effect on the LDH

content of the supernatant (Figure 3). The observation that the two compounds affected the tissues unequally led us to hypothesize that Troglitazone does not impair the cell membrane permeability, because it had only a slight effect on the LDH level in the supernatant. However, it clearly affects liver functionality, because it caused impairment of albumin secretion. In contrast, Staurosporine induces damage of the plasma membrane and therefore leakage of LDH and Albumin into the supernatant. The latter effect masked the impairment of Albumin secretion, which was only slightly detectable (Figure 3).

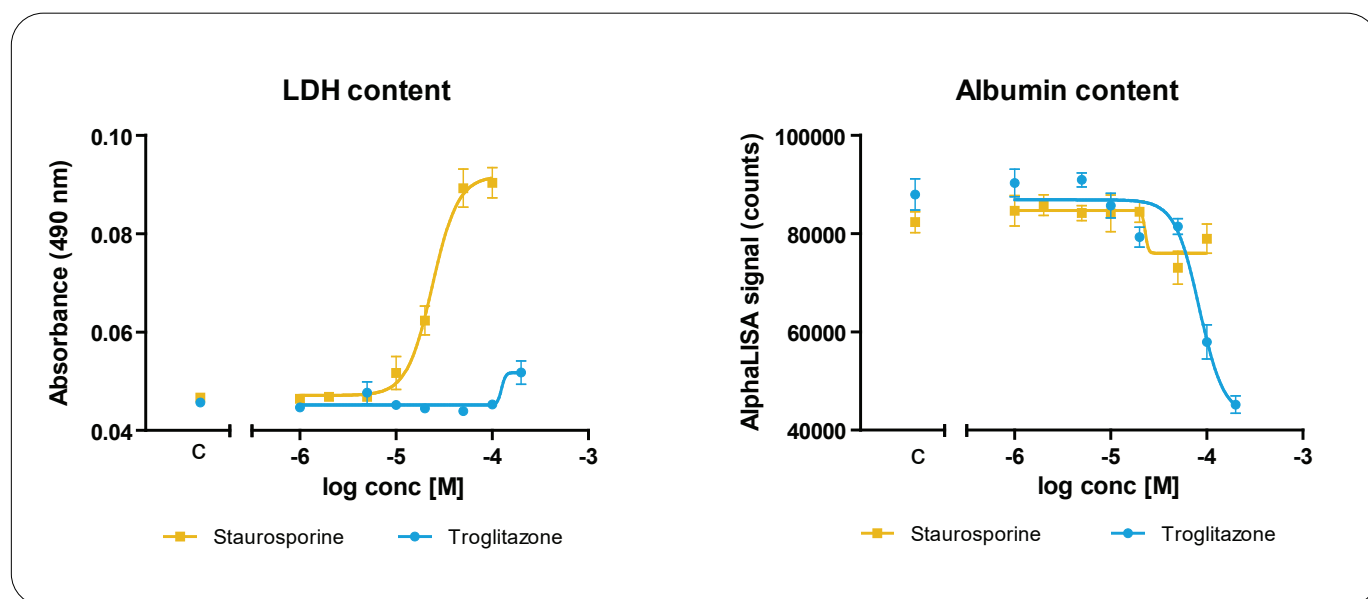


Figure 3: Evaluation of biochemical assays. The amount of Lactate Dehydrogenase (LDH) enzyme in the supernatant of microtissues increased with elevated cytotoxic compound concentrations. There is a distinct and dose-dependent release of LDH into the supernatant of the tissues when treated with Staurosporine and only a slight increase when treated with Troglitazone (left). The secretion of Albumin into the supernatant of treated microtissues is dose-dependently affected in Troglitazone treated tissues. Treatment with Staurosporine impaired albumin secretion only slightly (right).

Conclusions

In this study, we demonstrate a very effective assay for evaluating hepatotoxicity using complementary readouts. Using liver microtissues as a highly biologically-relevant 3D model system in combination with high-content imaging and biochemical assay detection technologies signifies a major step towards improving preclinical toxicity testing. On the one hand, the detection of LDH and Albumin secretion as metabolic readouts on the basis of supernatant analyses revealed metabolic changes in the liver microtissues. On the other hand, high content analysis of microtissues allowed for

investigation of cell integrity including different aspects of phenotypic changes. The extra value of the complementary readouts is highlighted by the inconsistent results obtained from the biochemical assays. In a small follow-up imaging experiment we were able to detect changes in the membrane texture of microtissues, further supporting our hypothesis on the different mechanisms of liver toxicity induced by different compounds. Additionally, automated liquid handling allows us to achieve low variability and long-term sterile assay conditions.

References

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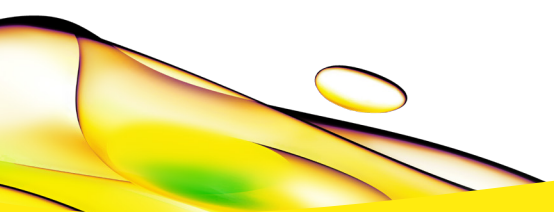
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