Combined High Content Imaging in Liver Microtissues and Mitochondrial Toxicity to Predict DILI

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INTRODUCTION

Drug-induced liver injury (DILI)

- DILI is a leading cause of attrition during drug development with mitochondrial toxicity as a considerable aetiology which has led to withdrawal of drugs such as troglitazone and cerivastatin[2]
- Previously, *in vitro* strategies focused primarily on 2D cellular models to evaluate DILI
- Newer strategies for the prediction of human DILI using primary human hepatocyte (PHH) 3D liver microtissues and utilizing ATP as an endpoint have been published[3),4)]
- Research at Cyprotex has expanded on this approach by using multi-parametric confocal High Content Screening (HCS) to detect MMP, ROS formation and GSH content in addition to cellular ATP in a co-culture human hepatocyte 3D model (hLiMTs) and a HepaRG human liver 3D model. Further analysis using a mitochondrial stress test has been combined with HCS.

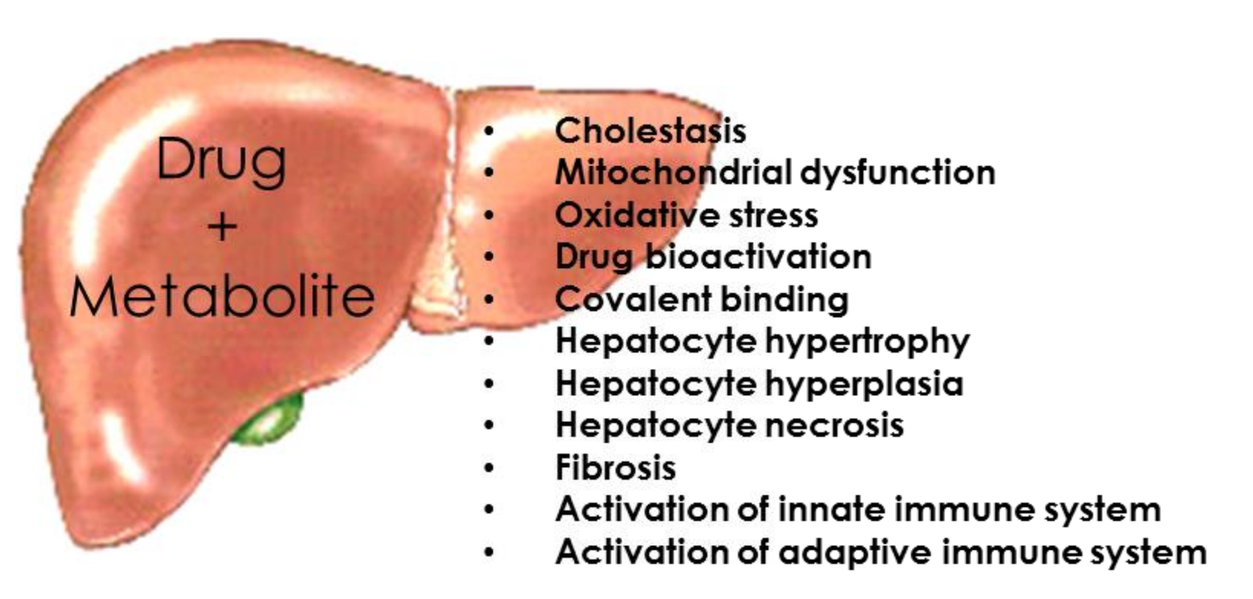

Figure 1: Mechanisms of DILI

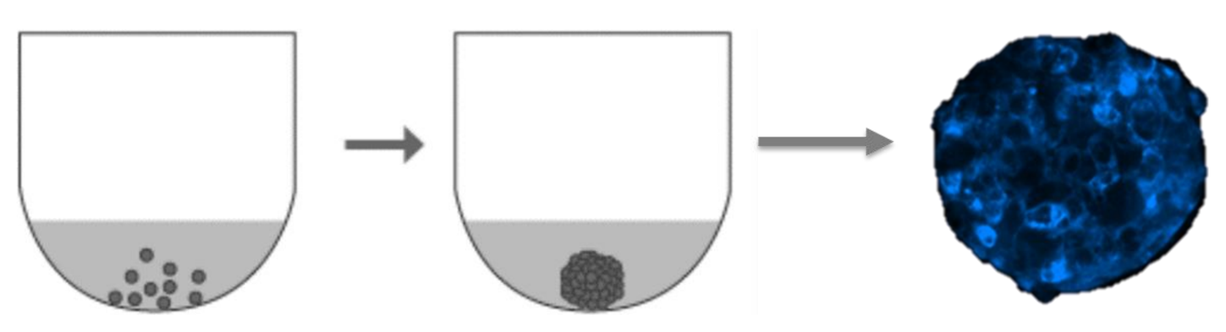

Figure 2: Microtissue or spheroid formation in ultra-low attachment (ULA) plates

AIMS

- Comparison of hLiMTs and HepaRG 3D microtissues (Cytochrome P450 expression and dose normalization)
- Determination of the predictive capabilities of HepaRG cells using a multiparametric high content screening approach (3D) combined with a mitochondrial stress test (2D)

METHODS/RESULTS

High content screening (HCS) assay design

- Microtissues were formed from cryopreserved human hepatocytes co-cultured with human non-parenchymal cells (hLiMTs) and HepaRG cells cultured as a spheroid model using scaffold free 96-well ultra low attachment round bottom plates (Greiner®)
- CYP activity was determined by incubating the microtissues/spheroids with probe substrates and the formation of CYP-specific metabolites was measured by LC-MS/MS
- Following exposure to a set of DILI-positive or DILI-negative compounds for 14 days, microtissues/spheroids were labelled with either Syto11 (DNA structure), monochlorobimane (mBCl) (GSH content), dihydroethidium (DHE) (ROS formation) and MitoTracker deep red (mitochondrial function) by incubation for 30 minutes
- Fluorescent images were acquired using the confocal mode of an ArrayScan™ XTI or CX7 HCS reader (ThermoScientific) in live cell mode followed by assessing cellular ATP (Promega)
- A mitochondrial stress test was carried out using a XFe96 Analyzer (Agilent) to measure oxygen consumption rate (OCR) and Extra Cellular Acidification Rate (ECAR) of live cells

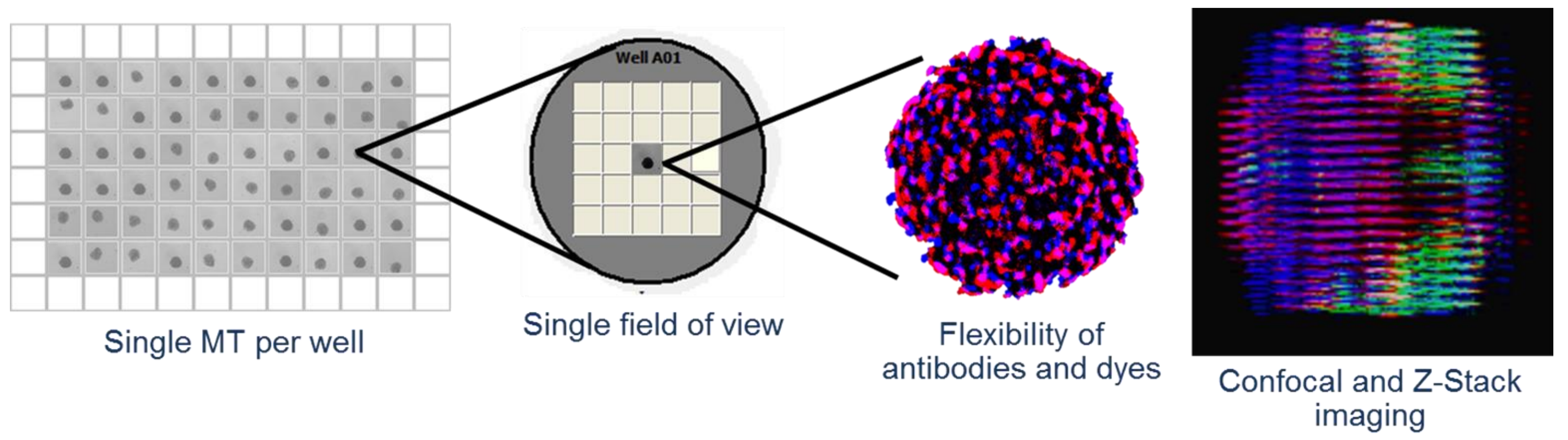

Figure 3: Principles of three dimensional (3D) confocal high content screening (HCS)

Representative 3D confocal HCS images of 3D microtissues

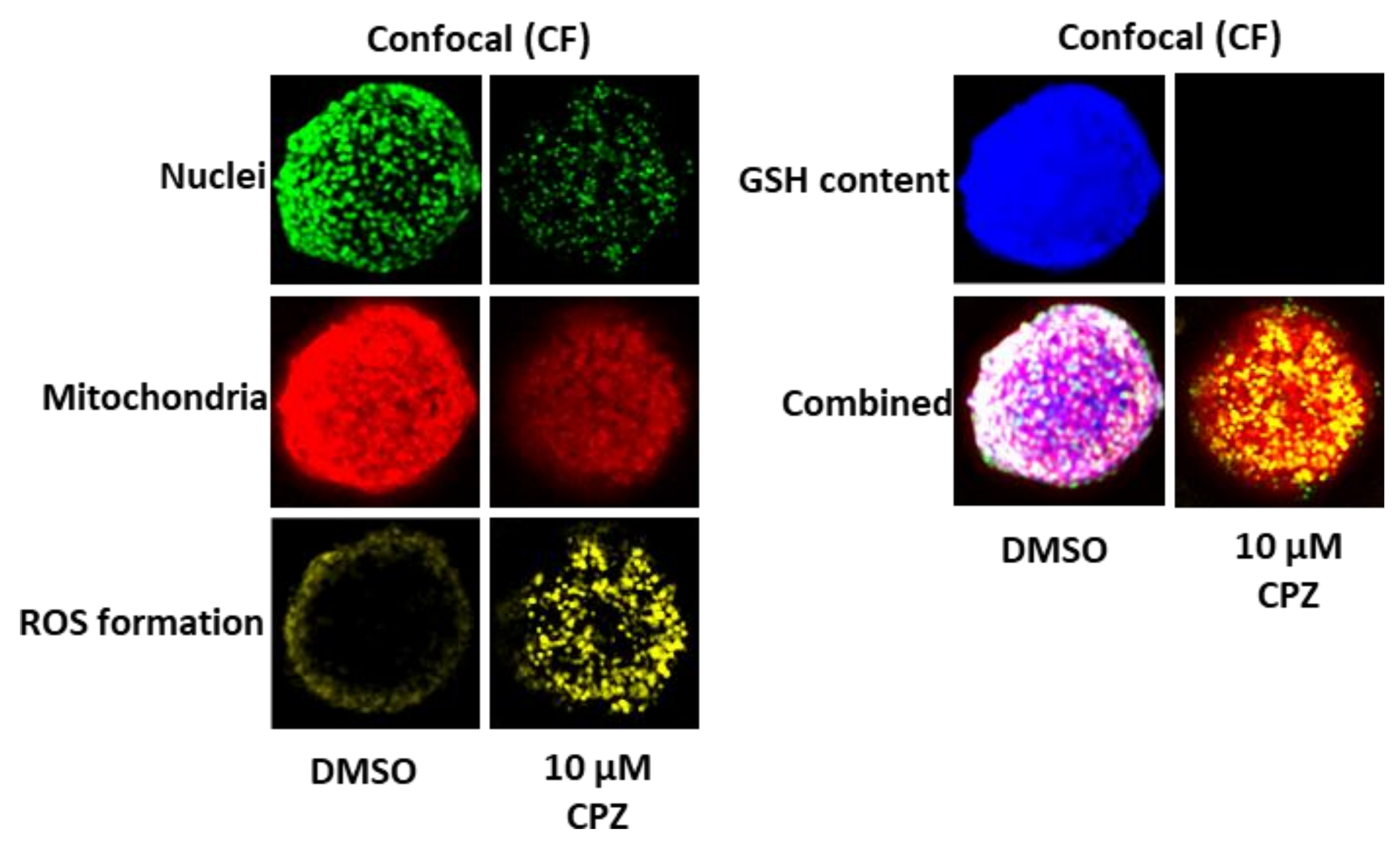

Figure 4: Representative 3D confocal high content screening (HCS) images of 3D microtissues labelled with Syto11 to detect DNA structure (green), monochlorobimane (mBCl) to detect GSH content (Blue), dihydroethidium (DHE) to detect ROS formation (yellow) and MitoTracker deep red to detect mitochondrial function (Red).

Comparison of hLiMTs and HepaRG spheroids

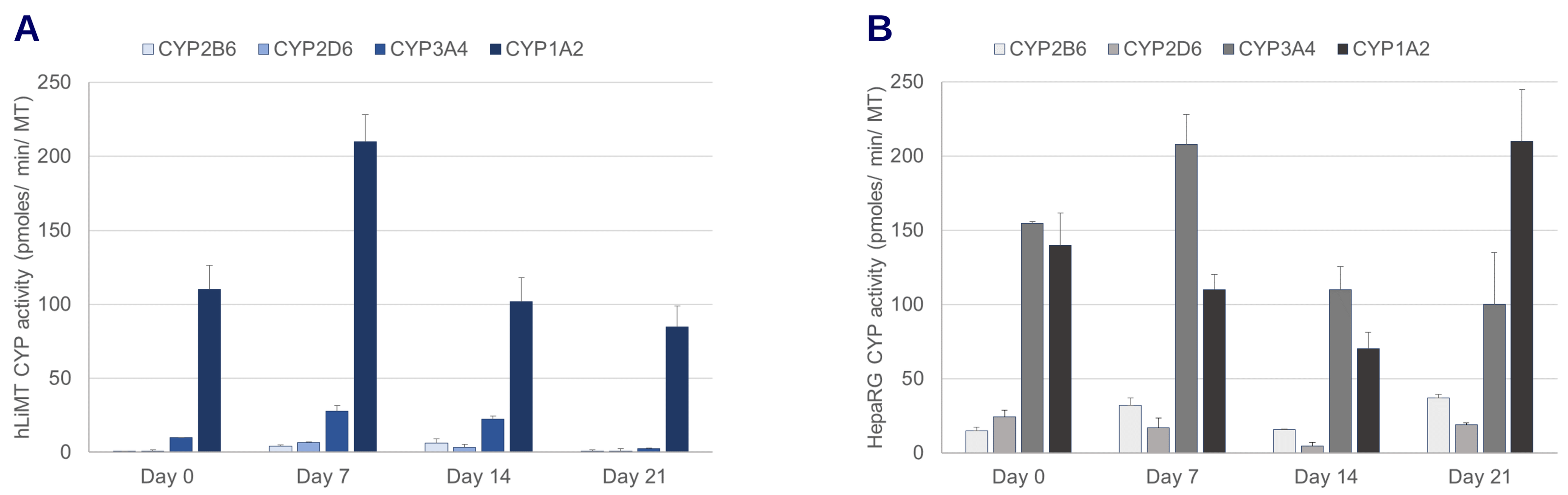

Figure 5: Characterisation of Cytochrome P450 (CYP) activity in (A). hLiMTs and (B). HepaRG microtissues over 21 days.

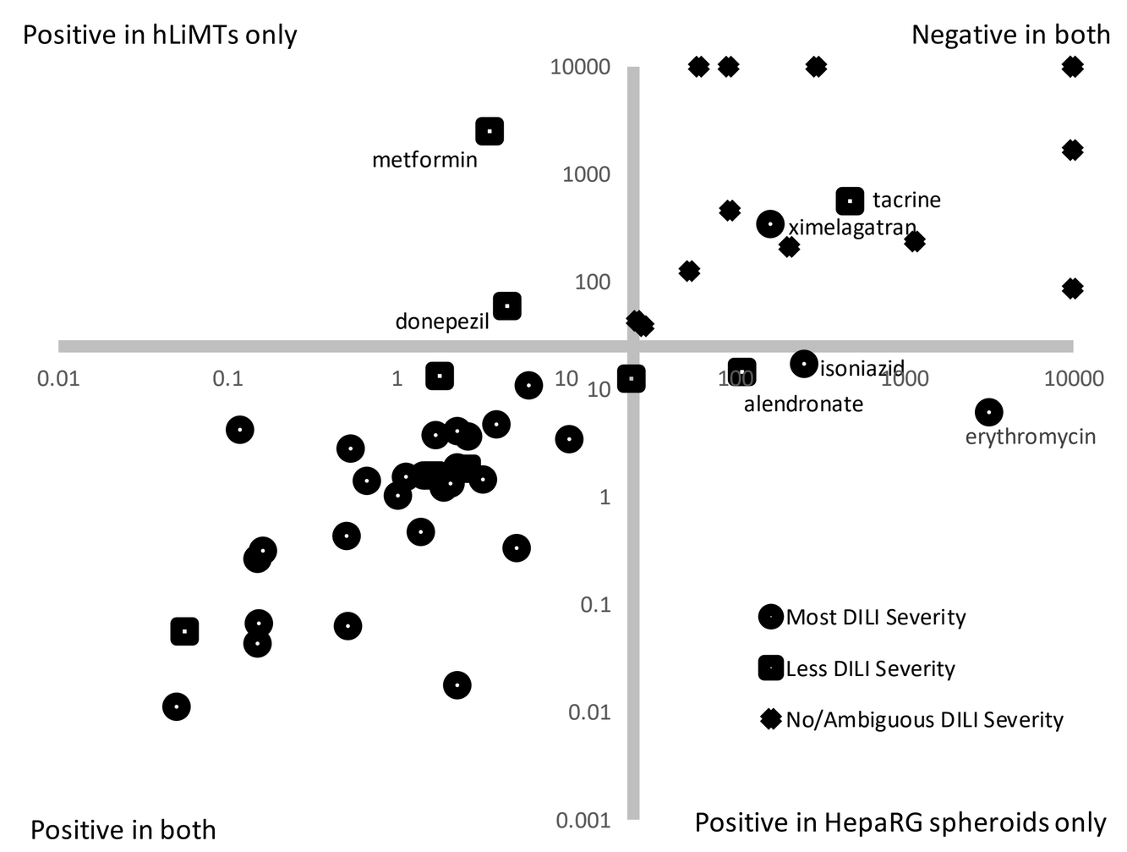

Figure 6: Comparison of therapeutic index (TI) for hLiMT's with hepaRG spheroids for 57 compounds with various known DILI risks. HepaRG spheroids plotted on the y-axis and hLiMT's plotted on the x-axis. Open circle, severe DILI potential; open square, moderate DILI potential; cross, low DILI potential. Axis crossing set at 25 to represent a 25x total plasma C_{max} cut off.

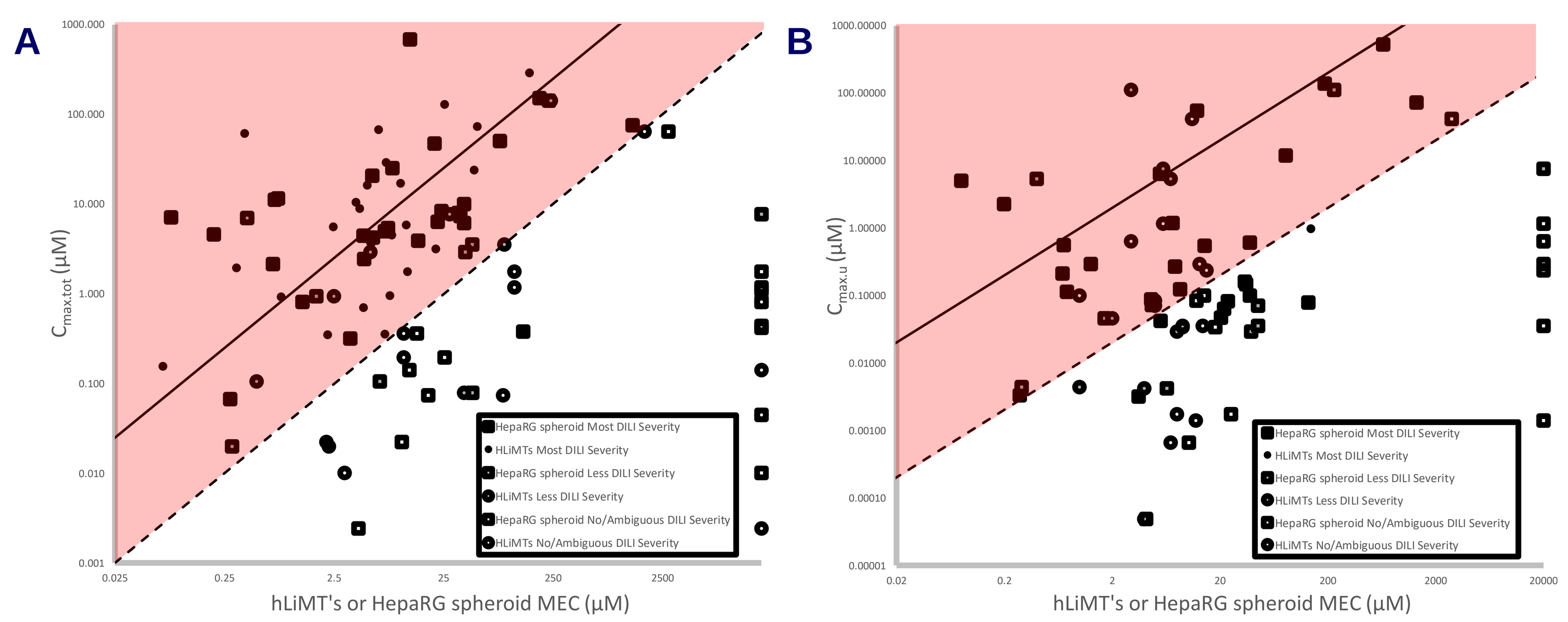

Figure 7: Correlation of hLiMT and hepaRG spheroid minimal effective concentration (MEC) with (A.) total plasma C_{max} or (B.) free $C_{max,u}$. Assigned DILI potential categories taken from Chen *et al.*, 2016[1] when available, otherwise average literature category used. Open circle, HepaRG spheroid severe DILI potential; closed circle, hLiMT severe DILI potential; open square HepaRG spheroid moderate DILI potential; closed square hLiMT's moderate DILI potential; line, HepaRG spheroid low DILI potential; cross, hLiMT's low DILI potential. Dashed line represents a 25x C_{max} or $C_{max,u}$ cut off. Red shading highlights area of positive DILI potential. Non-responding compounds assigned an arbitrary value of 1,000 µM.

Comparison of hLiMTs and HepaRG spheroids cont.

- Using a 25x C_{max} MEC cut-off was compared in hLiMTs vs HepaRG spheroids, with sensitivity of 87% and 89% respectively with 100% specificity for both
- The sensitivity and specificity of hLiMTs and HepaRG spheroids was determined relative to human dose, either total or free plasma C_{max} concentrations
- For the combined HCS and ATP assay the sensitivity of hLiMTs was 87% (total) and 61% (free). HepaRG sensitivity 89% (total) and 63% (free), indicating total plasma C_{max} dose normalisation is currently the best metric for IVIVE.

		Combined Assay (MEC/25xC_{max})	ATP only (MEC/25xC_{max})	HCS only (MEC/25xC_{max})	Combined Assay (MEC/100xC_{max},u)	ATP only (MEC/100xC_{max},u)	HCS only (MEC/100xC_{max},u)
hLiMTs	sensitivity	87%	71%	87%	61%	45%	55%
	specificity	100%	100%	100%	86%	93%	93%
	accuracy	91%	80%	91%	67%	58%	65%
HepaRG spheroids	sensitivity	89%	84%	84%	63%	50%	61%
	specificity	100%	100%	100%	93%	93%	93%
	accuracy	93%	89%	89%	71%	62%	69%

Table 1: Sensitivities & specificities in hLiMT's and hepaRG spheroids.

Screening of DILI-positive or DILI-negative compounds in HepaRG Spheroids

- 40 DILI-positive and 53 DILI-negative compounds were screened using HCS (3D HepaRG spheroids) and mitochondrial stress test (2D) in HepG2 cells.
- Results from these assays combined correctly identified 80 compounds as either DILI-positive or negative with only 4 compounds identified as FP and 9 compounds as FN.

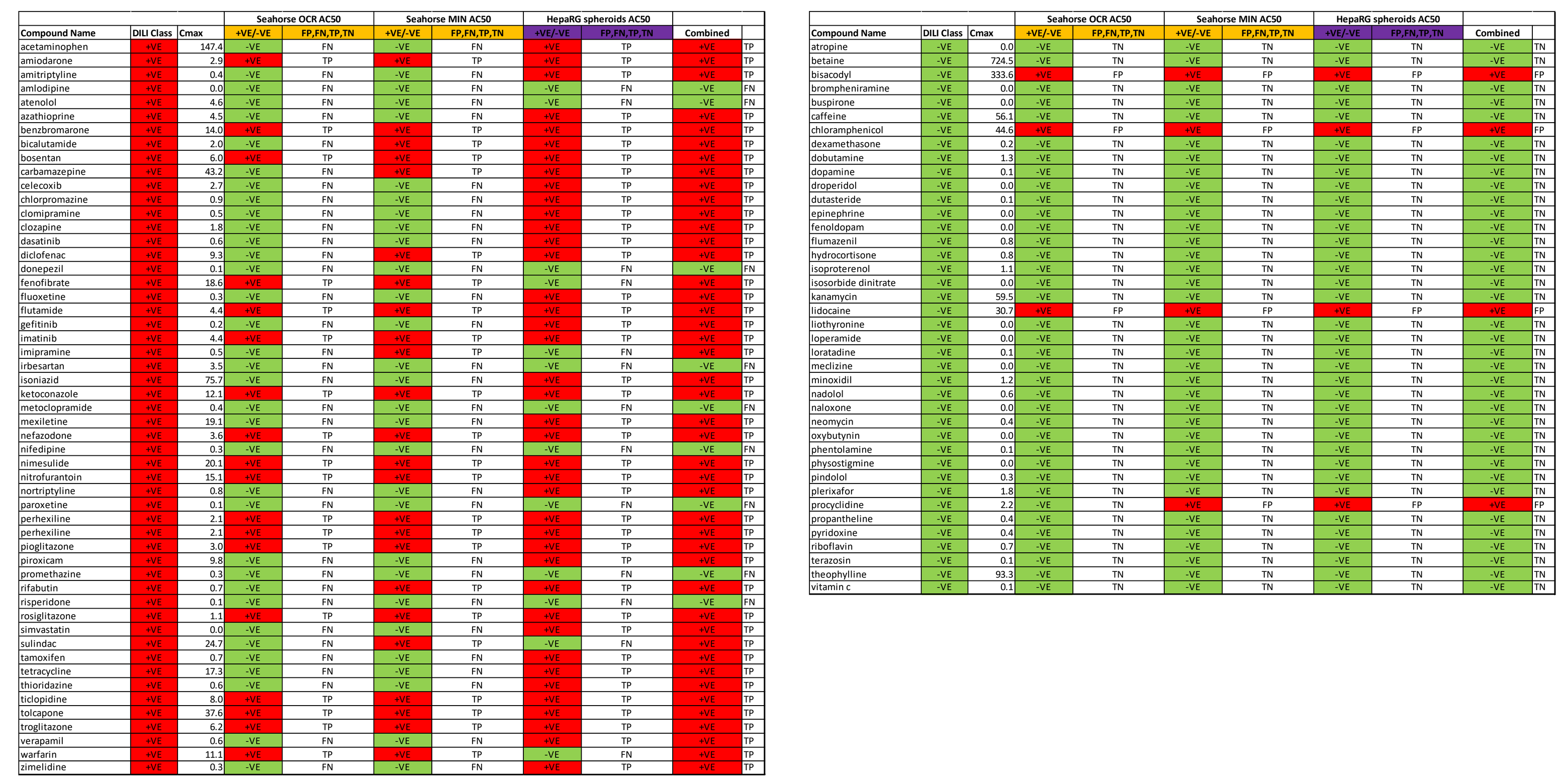

Table 2: Screening of 93 compounds known to be DILI-positive or DILI-negative in HepaRG spheroids and mitochondrial stress test.

SUMMARY/CONCLUSIONS

- Cyprotex have further developed the liver organoid approach by extending the number of endpoints using multiparametric HCS analysis in addition to cellular ATP content. Using this approach, we demonstrate a similar specificity but improved sensitivity over ATP content alone.
- HepaRG spheroids tend to have increased sensitivity over hLiMTs to predict DILI-positive compounds. This maybe due to increased level of CYP activity in the HepaRG spheroid compared to this individual donors from hLiMTs.
- Utilising HCS and a mitochondrial stress test we have correctly identified 80 compounds as either DILI-positive or DILI-negative in this combined approach
- Liver organoids are a valuable addition in the *in vitro* toolbox for de-risking DILI potential

REFERENCES

[1] Chen et al., 2016. DILIrank: the largest reference drug list ranked by the risk for developing drug-induced liver injury in humans. Drug Discovery Today 21: 648-653.
[2] Dykens JA and Will Y. 2007. the significance of mitochondrial toxicity testing in drug development. Drug Discovery Today 12: 777-785.
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[4] Vorrink, et al., 2018. Prediction of drug-induced hepatotoxicity using long-term stable primary hepatic 3D spheroid cultures in chemically defined conditions. Toxicol. Sci. 163(2), 655-665.