Utilization of Human-Focused 3D Liver Models in Conjunction with Multi-parametric Cell Health Approaches for the Prediction of Human Drug-Induced Liver Injury

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INTRODUCTION

Drug-induced liver injury (DILI)

- Drug induced hepatotoxicity is a leading cause of attrition during drug development.
- Until recently, *in vitro* strategies to evaluate DILI have focused primarily on 2D cellular models.
- In 2017, Genentech[1] published a new strategy for the prediction of human DILI using primary human hepatocyte (PHH) 3D liver microtissues. The approach compared 2D versus 3D models utilizing a single biochemical endpoint, ATP to reference DILI compounds.
- Our research at Cyprotex has developed this approach further by using a multi-parametric HCS approach to detect mitochondrial membrane potential, reactive oxygen species formation and GSH content in addition to cellular ATP in a co-culture human hepatocyte 3D model and a HepaRG human liver 3D model.

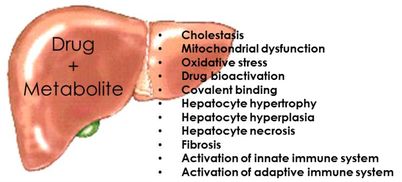

Figure 1. Mechanisms of drug induced liver injury (DILI)

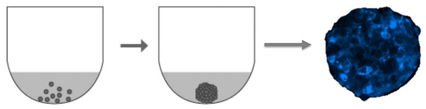

Figure 2. Microtissue formation in ultra-low attachment (ULA) plates

Aims

- Characterization of cytochrome P450 (CYP) levels in HepaRG 3D microtissues.
- Determination of the predictive capabilities of a co-culture human hepatocyte 3D microtissue model and HepaRG 3D microtissues using a multi-parametric approach and comparison with previous data evaluating ATP alone.

METHODS/RESULTS

High content screening (HCS) assay design

- Microtissues were formed from either cryopreserved human hepatocytes co-cultured with human non-parenchymal cells, or HepaRG cells as a monoculture model using scaffold free 96-well ultra low attachment round bottom plates (Corning®).
- CYP activity was determined by incubating the microtissues with probe substrates and the formation of CYP-specific metabolites was measured by LC-MS/MS.
- Following exposure to a reference set of DILI-positive and DILI-negative compounds for 14 days, microtissues 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 HCS reader (ThermoScientific) following with cellular ATP, which was measured using 3D CellTiter-Glo (Promega).

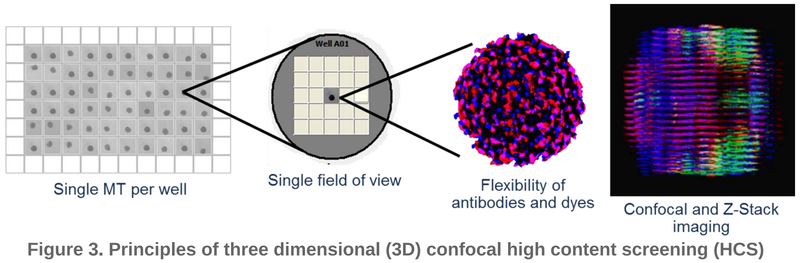

Figure 3. Principles of three dimensional (3D) confocal high content screening (HCS)

Cytochrome P450 activity in hLiMTs and HepaRG microtissues

Table 1: Cytochrome P450 activity in hLiMTs (pmol/ min/ million cells)

Enzyme	Metabolite	Day 0	Day 7	Day 14	Day 21
1A2	Acetaminophen	110±16	210±18	102±16	85±14
2B6	Hydroxybupropion	0.68±0.11	4.2±0.78	6.3±3.0	0.62±1.0
2D6	Dextrorphan	0.64±0.99	6.7±0.28	3.1±2.1	0.95±1.4
3A4	1-Hydroxymidazolam	9.7±0.08	27.8±3.6	23±2.1	2.7±0.17

Table 2: Cytochrome P450 activity in HepaRG microtissues (pmol/cmin/ million cells)

Enzyme	Metabolite	Day 0	Day 7	Day 14	Day 21
1A2	Acetaminophen	140±22	110±10	70±11	210±35
2B6	Hydroxybupropion	15±2.7	32±4.8	16±0.25	37±2.6
2D6	Dextrorphan	24±4.3	17±6.5	4.6±2.5	19±1.3
3A4	1-Hydroxymidazolam	155±1.3	208±20	110±15	100±35

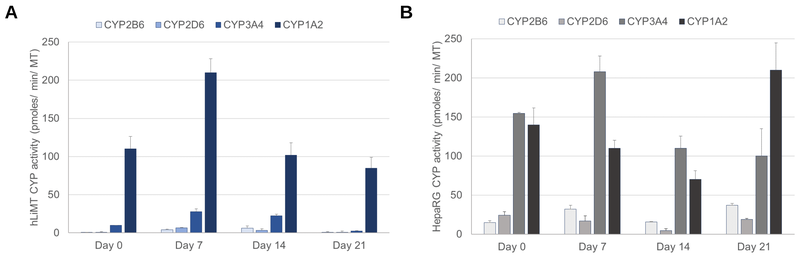

Figure 4. Characterization of cytochrome P450 (CYP) activity in (A). hLiMTs and (B). HepaRG microtissues over 21 days.

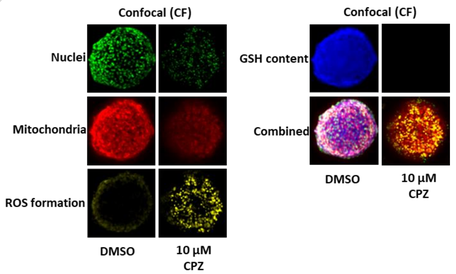

Figure 5. Representative 3D confocal high content screening (HCS) images of 3D microtissues labelled with Syto11 (green) to detect DNA structure, monochlorobimane (mBCl) (Blue) to detect GSH content, dihydroethidium (DHE) (yellow) to detect ROS formation and MitoTracker deep red (Red) to detect mitochondrial function.

Table 3. Assessment of the predictive capability of hLiMT and HepaRG 3D models for a set of 21 reference compounds.

Data was normalised to either 1x, 5, or >5x Cmax. The first response (MEC) was used from either cellular ATP alone or combined with HCS.

Drug	C_{max} (µM)	DILI category	Cyprotex hLiMT DILI prediction using MEC (µM)	Cyprotex hLiMT DILI prediction using ATP MEC (µM)	Most Sensitive Feature	Cyprotex 3D HepaRG spheroid DILI prediction using MEC (µM)	Cyprotex 3D HepaRG DILI prediction using ATP MEC (µM)	Most Sensitive Feature
Amiodarone	5.3	P	6.51	15.4	ROS	2.41	5.12	DNA
Trovafloxacin	19.7	P	45.2	54.4	GSH	7.27	7.27	ATP
Diclofenac	10.1	P	50	78.1	DNA	30.5	38.8	DNA
Flutamide	5.4	P	3.63	8.72	ROS	7.43	7.75	SIZE
Lapatinib	19.2	P	1.79	12.6	ROS	0.77	1.21	GSH
Nitrofurantoin	21	P	24.7	51.3	ROS	4.89	9.27	SIZE
Carbamazepine	50.8	P	49.2	73.6	DNA	81.5	81.5	ATP
Sunitinib	0.25	P	0.24	0.417	MMP	0.28	1.1	GSH
Troglitazone	6.29	P	0.99	1.55	MMP	1.69	25	DNA
Fialuridine	1	P	11.5	11.5	ATP	1.41	1.41	ATP
Nefazodone	4.3	P	13.7	13.7	ATP	11.6	11.6	DNA
Perhexiline	2.16	P	1.03	1.49	ROS	1.69	1.76	DNA
Tolcapone	21.96	P	21.9	21.9	ATP	18.2	20.5	MMP
Acetaminophen	165.4	P	302	302	ATP	240	342	SIZE
Bosentan	4.7	P	12.3	29.4	DNA	10.4	35.2	DNA
Ticlopidine	8.1	P	17.5	37.2	DNA	34	36.2	MitoMass
Azathioprine	2.22	P	2.48	2.48	ATP	0.28	0.278	ATP
Chlorpromazine	0.94	P	0.34	0.347	ROS	1.07	3.48	SIZE
Tamoxifen	1.18	P	1.54	1.98	ROS	3.52	10.8	SIZE
Buspirone	0.01	N	3.12	3.12	ATP	NR	-	-
Entacapone	3.276	N	40.2	40.2	ATP	45.4	45.5	GSH

≤ 1x Cmax
<5x Cmax
>5x Cmax

Comparison of 2D vs 3D and ATP vs multi-parametric HCS approach

Table 4: Illustration of the advantages of 3D models for the prediction of DILI as demonstrated by Proctor *et al.*, 2017 and further development of the approach by Cyprotex using a confocal high content imaging multi-parametric approach.

			3D hLiMTs (14d) ATP			2D PHH (48h) ATP			3D HepaRG MTs (14d) HCS		
			Proctor et al., 2017[1]			Proctor et al., 2017[1]			Cyprotex		
				Cutoff			Cutoff			Cutoff	
Compound	P/N	Cmax	IC50 (µM)	100µM	50xCmax	AC50 (µM)	100µM	50xCmax	AC50 (µM)	100µM	50xCmax
Entacapone	N	3.276	152.31	TN	FP	100.2	TN	FP	153	TN	FP
Buspirone	N	0.01	163	TN	TN	> 200.0	TN	TN	>200	TN	TN
Minoxidil	N	1.195	> 100.0	TN	TN	> 100.0	TN	TN	>100	TN	TN
Nadolol	N	0.808	> 100.0	TN	TN	> 100.0	TN	TN	>100	TN	TN
Neostigmine	N	0.045	> 10.0	TN	TN	> 10.0	TN	TN	>100	TN	TN
Mitomycin C	P	7.1	< 0.8	TP	TP	14.8	TP	TP	<0.4	TP	TP
Sunitinib	P	70.8	2	TP	TP	13.3	TP	TP	1.06	TP	TP
Tamoxifen	P	0.4	2.5	TP	TP	18.7	TP	TP	12.7	TP	TP
Perhexiline	P	2.16	2.6	TP	TP	11.2	TP	TP	3.15	TP	TP
Ketoconazole	P	11.3	4.6	TP	TP	47.2	TP	TP	8.42	TP	TP
Chlorpromazine	P	0.94	7.4	TP	TP	19.2	TP	TP	4.92	TP	TP
Troglitazone	P	6.4	14.6	TP	TP	> 100.0	FN	FN	11.1	TP	TP
Tolcapone	P	47.6	19	TP	TP	158.1	FN	TP	27.8	TP	TP
Azathioprine	P	7.2	22.2	TP	TP	> 100.0	FN	FN	0.48	TP	TP
Ticlopidine	P	8.1	22.5	TP	TP	> 100.0	FN	FN	4.02	TP	TP
Flutamide	P	5.4	25.7	TP	TP	73.0	TP	TP	14.9	TP	TP
Lapatinib	P	19.02	27.3	TP	TP	33.3	TP	TP	2.02	TP	TP
Nefazodone	P	4.3	29.4	TP	TP	36.2	TP	TP	15.2	TP	TP
Amiodarone	P	5.3	31.6	TP	TP	45.0	TP	TP	9.13	TP	TP
Diclofenac	P	10.1	61.4	TP	TP	> 500.0	FN	FN	66.7	TP	TP
Nitrofurantoin	P	21	69.1	TP	TP	29.4	TP	TP	11.6	TP	TP
Bosentan	P	7.43	93	TP	TP	> 200.0	FN	FN	74.7	TP	TP
Chlorpheniramine	P	0.044	94.2	TP	FN	> 100.0	FN	FN	67.9	TP	FN
Carbamazepine	P	50.8	267	FN	TP	> 500.0	FN	FN	159	FN	TP
Acetaminophen	P	165.4	572.8	FN	TP	4596.0	FN	TP	760	FN	TP
Acetylsalicylic acid	P	1110	> 1000.0	FN	FN	> 1000.0	FN	FN	1630	FN	TP
Cyclophosphamide	P	143	> 2000.0	FN	FN	> 2000.0	FN	FN	419	FN	TP
Dantrolene	P	3.95	> 100.0	FN	FN	> 100.0	FN	FN	40	TP	TP
Indomethacin	P	8.4	> 100.0	FN	FN	> 100.0	FN	FN	94.2	TP	TP
Metformin	P	7.742	> 100.0	FN	FN	> 100.0	FN	FN	>1000	FN	FN
Methotrexate	P	4.995	> 100.0	FN	FN	> 100.0	FN	FN	<0.2	TP	TP
Trovafloxacin	P	5.02	> 125.0	FN	FN	> 125.0	FN	FN	12.9	TP	TP
Valproic acid	P	693.4	> 100.0	FN	FN	> 100.0	FN	FN	41.3	TP	TP
			TP	18	19		11	13		23	26
			TN	5	4		5	4		5	4
			FP	0	1		0	1		0	1
			FN	10	9		17	15		5	2
			Sens	64.3	67.9		39.3	46.4		82.1	92.9
			Spec	100.0	80.0		100.0	80.0		100.0	80.0

Improved sensitivity and specificity was observed using HepaRG MTS and a combined GSH, ROS, MMP and ATP assay versus hLiMTs or primary human hepatocytes with a cellular ATP endpoint. This data implies 3D approaches are ideally suited to de-risk drug discovery programs in comparison to the traditional 2D approaches using human hepatocytes.

SUMMARY/CONCLUSIONS

- A recent publication from Genentech[1] has demonstrated the value of 3D models over 2D approaches.
- Cyprotex have further developed the Genentech approach by extending the number of endpoints using multi-parametric HCS analysis in addition to cellular ATP content. Using this approach, we demonstrate a similar specificity but significantly improved sensitivity over ATP content alone.
- If comparing HepaRG microtissues to hLiMTs, the HepaRG microtissues tended to have increased sensitivity to predict DILI-positive compounds. This is likely to be a result of the increased level of CYP activity in this microtissue compared to the hLiMT model. This CYP activity in hLiMTs has been shown previously to vary considerably depending upon the donor (data not shown).

REFERENCES

[1]Proctor WR *et al.*, (2017) *Arch Toxicol* **91(8);** 2849-2863.