Use of complex hepatic models for a fit-for-purpose metabolism assessment of ingredients for use in Next Generation Risk Assessment

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Introduction

Next Generation Risk Assessment (NGRA) integrates estimations of internal exposure (e.g. plasma Cmax) with measures of bioactivity detected across a range of in vitro bioassays, to inform the safety of a new chemical for a defined consumer use scenario. Within this framework, there is a need to consider metabolism-driven toxicity. One of the bioassays used is the Cell Stress Panel (CSP), consisting of 36 biomarkers representing mitochondrial toxicity, cell stress, and cell health, measured predominantly using high content imaging.

One of the limitations of the CSP, as the assay is primarily used as a first screening assay, is that the cell line selected (HepG2) is typically exposed to chemicals for 24 hours. This timeframe might not be long enough to demonstrate that Point of Departures (PoDs) are influenced by metabolism of the chemical tested. Therefore, a Metabolism framework has been proposed to estimate at which point in the Risk Assessment of the parent chemical more complex tools might be required in the experimental design to demonstrate the formation of metabolites. Whether these are reactive metabolites, generally associated with toxicity in the tissue where they are formed, or stable metabolites with the potential to be toxic in several organs and cells (off target effect), simulating in vivo relevant exposure to metabolites when dosing with parent chemical in in vitro assays remains challenging.

The gold standard for studying liver metabolism in humans is still primary hepatocytes. However, due to the limited availability of primary cells, inter donor variability and the limited time for which incubation with test chemicals can be carried out, new in vitro tools and models, all covering hepatic metabolism, have been developed in the last few years. We selected two 3D HepaRG based systems to study chemicals based on our own Metabolism Framework for Risk Assessment. The first one is a co-culture model using a 3D toroid of HepaRG cells and the second one is a simplified CSP assay that can be used with HepaRG spheroids.

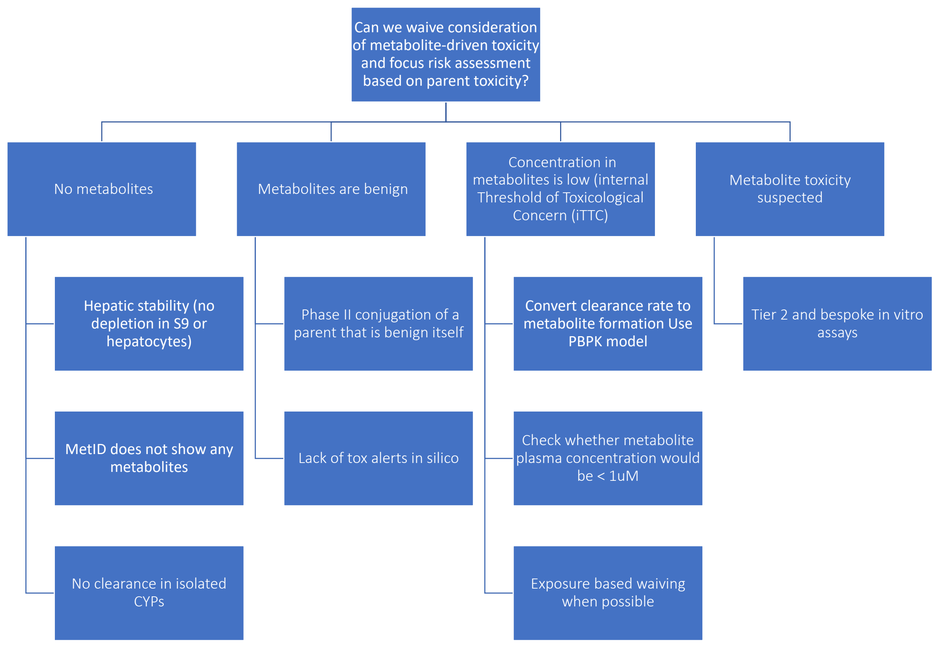

Fig.1. Metabolism considerations in Risk Assessment. Tier 1 assessment: Proposed experimental design to demonstrate that a risk assessment based on parent is appropriate and conservative (under evaluation).

Microphysiological liver model using HepaRG 3D toroid and AR-CALUX cells

It has been demonstrated that the HepaRG cell line exhibits a metabolic competency higher than other liver cell line such as HepG2 both in 2D culture and in 3D culture. As part of a collaboration with Brown University, we have investigated whether HepaRG cells culture in 3D in a complex structure (allowing for a higher number of cells than a standard spheroid culture) would allow to scale-up metabolic transformation of parent compound into metabolites demonstrating a toxicological an effect on a target cell line.

Here, HepaRG cells were incubated on the outside ring of a well filled with an agarose matrix, with cells from the stably transfected human osteosarcoma (U2OS) cell line expressing the human AR (AR-CALUX cells) used as a toxicity target on the inside chamber of the well. Using this set-up for HepaRG cells, CYP activity was demonstrated in the toroid for CYP1A1, CYP1A2, CYP2B6, CYP2C9, and CYP3A4 and the impact of testosterone metabolism was visible on the AR-CALUX cells as reduced testosterone-mediated activation of androgen receptor (AR) was observed.

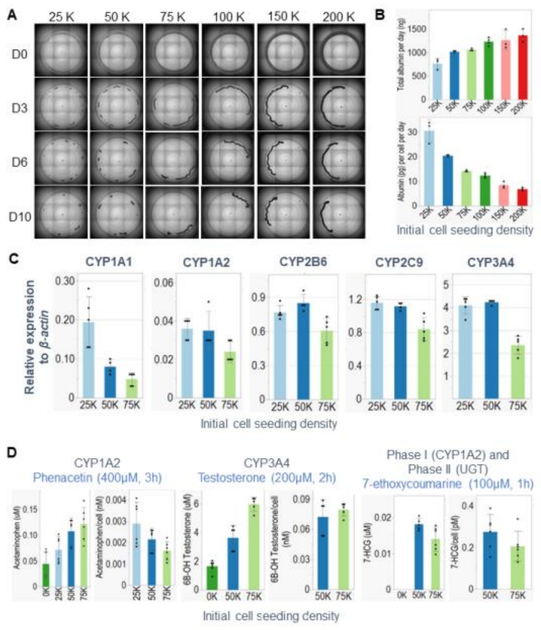

Fig.2. Optimization of HepaRG seeding densities in two-chamber systems. Seeding densities of HepaRGs from 25,000 to 200,000 cells per well were examined in the two-chamber systems. A) Representative live cell brightfield images showing HepaRG cells forming 3D microtissues during culture in the two-chamber system for 0, 3, 6 and 10 days. B) Graphical representation of total albumin secreted per day (ng) and the corrected secreted albumin per number of cells initially seeded (pg/d) after 10 days of maturation, showing that increasing cell seeding density decreased albumin secretion on a per cell basis. C) Decline in hepatic Phase I CYP enzyme gene expression of HepaRG 3D microtissues matured for 10 days when the initial seeding density increased from 50,000 to 75,000 cells per well. D) Decline in hepatic Phase I/II enzyme function of HepaRG 3D microtissues matured for 3 days (phenacetin) or 10 days (testosterone; 7- ethoxycoumarin) when the initial seeding density increased from 25,000 or 50,000 to 75,000 cells per well.

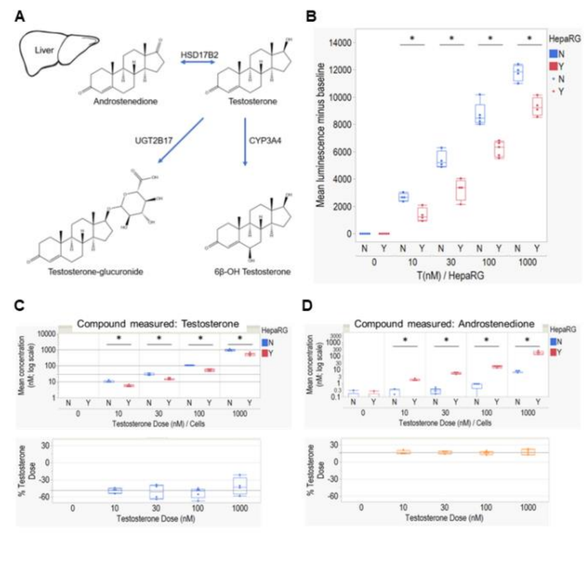

Fig.3. HepaRG 3D microtissues metabolized testosterone and reduced testosterone-mediated activation of androgen receptor (AR). Simplified metabolic pathway of testosterone biotransformation in the liver (A). Mean luminescence minus baseline of AR-CALUX reporter cells co-cultured with or without HepaRG 3D microtissues after incubation with testosterone (T) at 0, 10, 30, 100, or 1000 nM for 24 hours (B). Mean media testosterone concentration in nM (top) of wells with or without HepaRG 3D microtissues after incubation with testosterone (T) at 0, 10, 30, 100, or 1000 nM for 24 hours, and as % of starting testosterone doses (bottom) in wells with HepaRG (C). Mean media androstenedione concentration in nM (top) of wells with or without HepaRG 3D microtissues after incubation with testosterone (T) at 0, 10, 30, 100, or 1000 nM for 24 hours, and as % of starting testosterone doses (bottom) of wells with HepaRG (D). Data = mean ± SD. Student's t-test was used to examine statistical significance for the effects of HepaRG on AR-CALUX activation and compounds in media measured by LC-MS for each of the four doses independently.

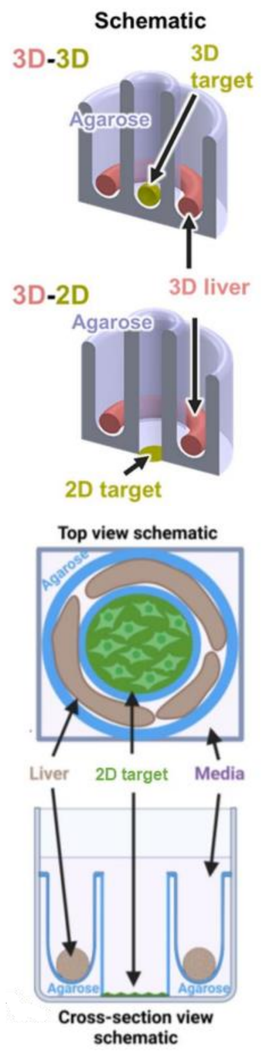

Fig.4. Schematic of 3D co-culture in agarose gel moulds, showing 3D toroid of HepaRG cells on the outer ring and 2D AR-CALUX cells as a target for metabolites in the centre of the mould.

Comparison of HepG2 2D, HepaRG 2D and 3D cultures for assessment of reactive metabolites

In collaboration with Cyprotex, we are carrying out a simplified Cell Stress Panel assay on HepG2 2D cultures and HepaRG 2D cultures after a single (24h) or double treatment regimen (72h) and comparing with a multi-exposure regimen on 3D HepaRG spheroids (5 doses over a 14-day period). The cells are dosed with test article at a range of concentrations and incubated for various time points (24 hr and 72 hr for HepG2 and HepaRG; 24 hr, 72 hr, 168 hr and 336 hr for HepaRG spheroids).

At the end of the incubation period, the cells are loaded with the relevant dye for each cell health marker. The plate designs provide simultaneous measurement of multiple cell health parameters for each of the multiparameter HCS assays: GSH ROS MMP ATP & LDH assay, PLD & Steatosis assay, DNA damage assay (phospho-p53 and pH2AX), Mitochondrial Potential (TMRE) as well as cell count, nuclear size and DNA structure for 2D cell culture or spheroid count, spheroid size and DNA structure for 3D cell cultures. The plates are scanned using an automated fluorescent cellular imager, ArrayScan® VTI or XTI or CX7 (Thermo Scientific Cellomics).

These targets have been selected to be predictive for compounds causing liver toxicity but also potentially other forms of toxicity.

Chemical ID	CAS number	MW (g/mol)	Reactive metabolite of interest (a)	Cytotoxicity top dose in assay (µM) (b)
Diclofenac [sodium salt]	15307-79-6	318.13	Quinoneimine (after CYP formation of 5-hydroxydiclofenac)	500
Acetaminophen	103-90-2	151.16	NAPQI (quinoneimine)	2000
Sunitinib [malate]	341031-54-7	532.56	Quinoneimine (formed after CYP induced oxidative defluorination)	200
Fialuridine	69123-98-4	372.09	Metabolites generated via CYPs	500
Troglitazone	97322-87-7	441.54	Quinone and o-quinone methide	200
Ketoconazole	65277-42-1	531.43	Reactive metabolite via CYP3A4	500
Cyclophosphamide	6055-19-2	279.1	Phosphoramide mustard	2000
Eugenol	97-53-0	164.2	Quinone type	500
Methyl eugenol	93-15-2	178.23	Non-reactive metabolite	500
Hydroquinone	123-31-9	110.11	Quinone	200
Retrorsine	480-54-6	351.39	Dehydroretrorsine via CYP3A4	500
4-Hexylresorcinol	136-77-6	194.27	Quinone type	500

Table.1. Chemical selection and top dose concentrations to assess cytotoxicity in pre-screening assays. (a) Chemicals have been selected for their potential to form reactive metabolites in situ. These metabolites can react with GSH or DNA and or induce ROS, all these biomarkers being included in the simplified CSP. (b) Top doses applied in a pre-screening assay for assessing cytotoxicity of compounds on HepG2 and HepaRG cells as well as HepaRG spheroids. Internal BIFROST model is used to derive a PoD (Point of departure) for cytotoxicity and select top concentrations to be included in the simplified CSP.

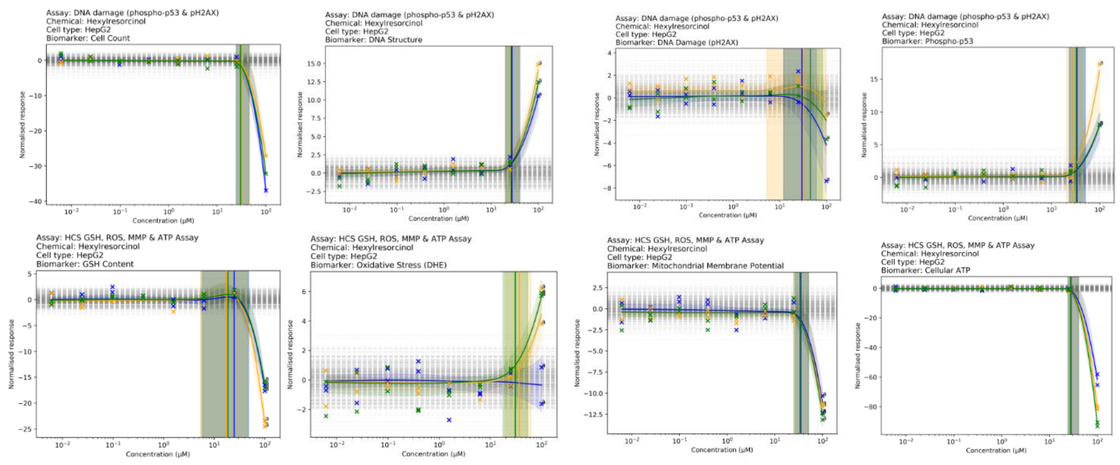

Fig.5. Example of results obtained for HepG2 cells treated with 4-Hexylresorcinol for 24h. Raw data are analysed using our internal BIFROST model to generate dose-response curves and PoDs for each biomarker. Top row, left to right: Cell count, DNA structure, pH2AX (DNA damage) and Phospho-p53 (DNA damage) from the DNA damage plate. Bottom row, left to right: GSH, Oxidative stress (ROS), MMP and ATP.
Overall, the biomarkers selected do not show any significant activity before cell loss occurs due to cytotoxicity (LDH PoD is 46µM at 95th percentile, data not shown) and the dose-response curves have confidence scores of between 0.6 and 1.0. A complementary set of data using the HepaRG cell line (in 2D or 3D culture, covering multiple treatments) might demonstrate the effect of 4-hexylresorcinol metabolism and show variations in PoDs (data currently generated).

Conclusion

We have used two complex in vitro liver models to study the impact of metabolism on bioactivity/toxicity in addition to the more traditional assays using short term incubations of cryopreserved primary human hepatocytes in suspension. Our conclusion is that the HepaRG cell model is proving a useful tool to study metabolism of single chemicals in vitro.

References

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