High-content imaging for the detection of compound reactive metabolite formation and cytotoxicity

David Cerny, Benjamin Park, Ryan Barton, Caroline Bauch, Samantha Bevan, Phillip Wright & Paul Walker

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Introduction

- Drug-induced liver injury (DILI) is one of the most common mechanisms for drug attrition in development, and market withdrawal of approved drugs
- The cytochrome P450 (CYP450) superfamily of enzymes form part of the phase 1 metabolic system within the liver; responsible for the oxidation, peroxidation and hydrolysis of drugs for further excretion
- For certain chemical entities, the CYP450 enzymes can adversely catalyze the formation of highly reactive metabolites, capable of cytotoxicity
- Such compound bioactivation can induce multiple adverse hepatotoxic response, including drug-related mitochondrial dysfunction, reactive oxygen species (ROS) formation, endoplasmic reticulum stress and DNA damage[1]
- 1-Aminobenzotriazole (ABT) is pan-specific inhibitor of the CYP450 enzymes and has been used to investigate their participation in metabolism
- Here we present a multiparametric high-content imaging method utilising ABT to determine bioactivation potential of drug candidates using metabolically competent cells and analysing key cell health features

Material and methods

Cell Culture

- HepaRG cells were seed onto collagen coated 96-well plates and allowed to adhere for 24hrs. The HepaRG culture was maintained for 7 days prior to compound treatment with media changes every 72hrs
- Primary human hepatocytes (PHH) and primary rat hepatocytes (PRH) were seeded onto collagen coated 96-well and allowed to adhere for 6hrs after which a matrigel (Corning) sandwich culture was applied at a concentration of 0.25mg/ml

Compound Selection

- 17 compounds were selected (Table 1), including 14 compounds with reported reactive metabolite formation and 3 without reported reactive metabolite formation
- 11 of the 14 compounds with known reactive metabolism undergo phase 1 enzyme metabolism and the remaining three compounds undergo CYP450 independent metabolism

		CYP450 Enzyme Metabolism										
Compound	Reactive metabolite formation	1A2	2A6	2B6	2C8	2C9	2C18	2C19	2D6	2E1	3A4	3A5	No Phase 1
acetaminophen	Reported									O			
aflatoxin B1	Reported	O									O		
carbamazepine	Reported			O	O						O	O	
clozapine	Reported	O				O		O	O		O		
cyclophosphamide	Reported		O	O		O	O	O			O	O	
flutamide	Reported	O									O		
ketoconazole	Reported								O		O		
methapyrilene	Reported												O
mifepristone	Reported				O	O					O		
sertraline	Reported			O		O		O	O		O		
tacrine	Reported	O											
tolcapone	Reported												O
trovafloxacin	Reported												O
valproic acid	Reported		O	O		O						O	
carbonyl cyanide 3-chlorophenylhydrazone	No Report												O
rotenone	No Report												O
staurosporine	No Report												O

Table 1: Validation compound set with reported or unreported reactive metabolite formation and corresponding CYP450 enzyme metabolism

Compound Treatment and Assay

- Compounds were treated both in the presence, and absence, of 1mM ABT in triplicate across 7 concentrations for 48hrs
- Post compound treatment cells were loaded with dyes for nuclei (Hoechst 33342), glutathione (monochlorobimane), reactive oxygen species (dihydroethidium) and mitochondria (MitoTracker Deep Red FM). Plates were scanned using the Cellomics ArrayScan HCI reader
- Cellular ATP content was assessed using CellTiter-Glo® (Promega) post HCI reading
- Fold-shifts were calculated according to either the AC_{50} or minimum effective concentration (MEC) against the +ABT and -ABT treatment conditions

References

1. Gómez-Lechón, M. J., Tolosa, L., and Donato, M. T. (2016) Metabolic activation and drug-induced liver injury: *in vitro* approaches for the safety risk assessment of new drugs. *J. Appl. Toxicol.*, **36:** 752– 768. doi: 10.1002/jat.3277

Results

- We first assessed the cellular ATP content in HepaRG, PHH and PRH cell models after treatment with aflatoxin B1 and methapyrilene in the presence and absence of ABT (Figure 1) to determine reactive metabolite formation
- The degree of metabolite induced toxicity was determined according to fold-shift calculated by AC_{50} values in each treatment condition, outlining aflatoxin B1 HepaRG: 25.7 (Figure 1A), aflatoxin b1 PHH: 22.9 (Figure. 1B), methapyrilene PRH: 6.88 (Figure 1C) and methapyrilene HepaRG: 0 (Figure 1D)

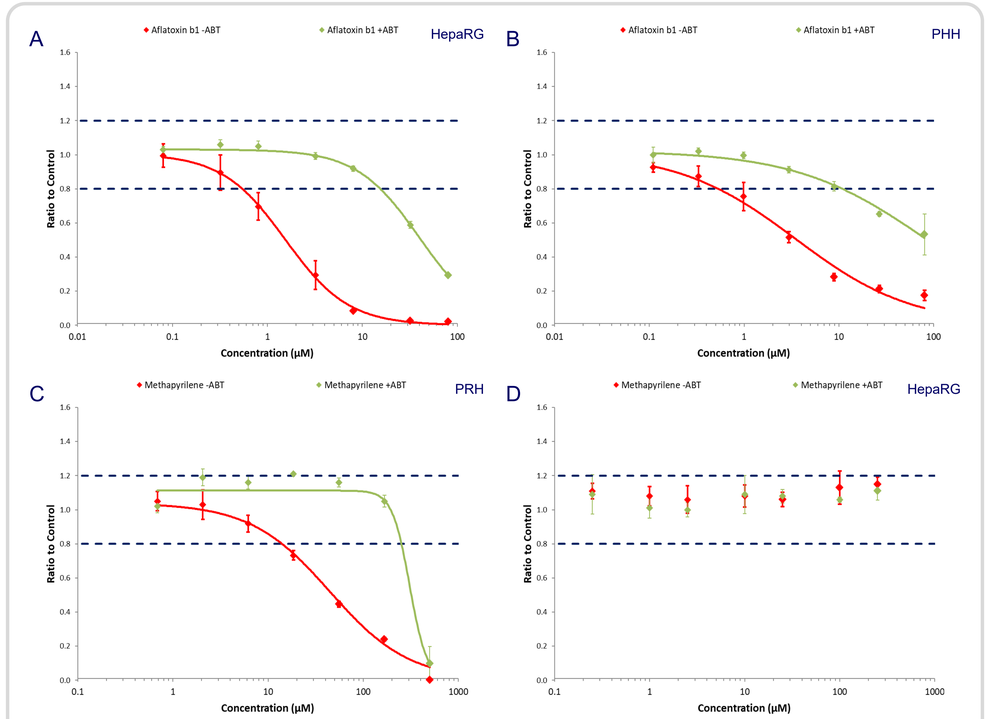

Figure 1: Cellular ATP content measurement post aflatoxin B1 and methapyrilene treatment in the cell models HepaRG, PHH, PRH. Cytotoxicity was calculated according to the AC_{50} fold-shift. Aflatoxin B1 treated HepaRG fold-shift 25.7 (1A), aflatoxin B1 PHH fold-shift 22.9 (1B), methapyrilene PRH fold-shift 6.88 (1C) and methapyrilene HepaRG fold-shift 0 (1D).

- We next employed a multiparametric high-content imagine (HCI) methodology to screen a series of compounds (Table 1) through the HepaRG cell model with fold-shift being calculated according to both MEC and AC_{50} values (Table 2 & Table 3)
- A cut-off value of 1.64 was applied to the MEC calculated fold-shift values of all measured cell health parameters (Table 2), providing 7 correctly identified positive compounds, 3 correctly identified negative compounds and 7 incorrectly identified compounds

		Cell Health Parameter MEC Fold-Shift							
Compound	Experimental Formation	Cell Count	Nuclear Area	DNA Structure	GSH Content	Oxidative Stress	Mitochondrial Mass	Mitochondrial Membrane Potential	Cellular ATP
Acetaminophen	Reported	0.861	0.778	1.678	UD	0.570	0.000	1.313	0.755
Aflatoxin b1	Reported	16.446	UD	23.153	UD	UD	8.400	UD	29.541
Carbamazepine	No Report	0.000	0.000	0.000	0.000	0.000	UD	0.000	0.000
Clozapine	Reported	0.000	0.000	1.692	1.026	1.333	0.000	2.680	1.012
Cyclophosphamide	No Report	0.000	0.000	UD	0.000	0.000	0.000	0.000	0.000
Flutamide	Reported	0.571	2.143	1.199	2.400	0.749	0.000	1.196	0.670
Ketoconazole	Reported	1.020	0.935	2.331	1.111	0.927	0.000	1.350	0.995
Mifepristone	No Report	0.288	0.758	0.826	1.558	0.470	0.000	0.575	0.875
Sertraline	Reported	1.024	0.812	1.975	0.944	0.445	0.000	0.956	1.067
Tacrine	Reported	0.000	0.000	1.207	1.641	0.000	0.000	1.213	UD
Valproic acid	No Report	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Carbonyl cyanide 3-chlorophenylhydrazone	No Report	0.823	0.681	0.796	0.841	0.389	0.000	0.512	0.608
Methapyrilene	No Report	0.000	0.000	UD	0.000	0.000	0.000	0.000	0.000
Rotenone	Reported	0.647	0.400	0.395	27.438	1.048	0.000	5.056	UD
Staurosporine	Reported	0.713	1.655	1.069	UD	0.763	UD	1.243	0.730
Tolcapone	Reported	1.213	0.775	0.963	1.040	1.962	0.000	0.000	0.744
Trovafloxacin	No Report	0.000	0.000	0.688	1.594	0.000	0.000	0.000	0.710

Table 2: Fold-shift calculated according to MEC values for each respective cell health parameter. Undetermined (UD) references when an MEC was not reported for one of the ABT treatment conditions. Compounds which did not generate an MEC value in either ABT treatment condition recorded a fold-shift of 0. A 1.64 cut-off was applied to facilitate toxic metabolite identification (highlighted green).

- A cut-off value of 1.25 was applied to the AC_{50} calculated fold-shift values of all measured cell health parameters, providing 4 correctly identified positive compounds and 13 incorrectly identified compounds

		Cell Health Parameter AC50 Fold-Shift							
Compound	Experimental Formation	Cell Count	Nuclear Area	DNA Structure	GSH Content	Oxidative Stress	Mitochondrial Mass	Mitochondrial Membrane Potential	Cellular ATP
Acetaminophen	No Report	0.474	0.786	0.457	0	0.72	1.05	UD	0.883
Aflatoxin b1	Reported	13.7	UD	64.2	0.456	UD	UD	16	25.7
Carbamazepine	No Report	0	0	UD	UD	UD	0	0	UD
Clozapine	No Report	0	0	0.506	0	0.805	0.71	1.07	0.994
Cyclophosphamide	No Report	0	0	UD	0	UD	0	0	0
Flutamide	Reported	0.697	1.25	0.924	UD	1.36	0.777	2.67	0.709
Ketoconazole	Reported	1.06	0.831	0.975	UD	1.3	0.853	0.843	0.918
Mifepristone	No Report	0.62	0.717	0.809	0	0.844	0.53	0.963	0.246
Sertraline	Reported	1.39	0.864	0.966	0	1.1	1.64	0.992	1.03
Tacrine	No Report	0	0	1.03	0	0	0	UD	UD
Valproic acid	No Report	0	0	UD	0	0	0	0	0
Carbonyl cyanide 3-chlorophenylhydrazone	No Report	0.852	0.827	0.46	0	0.867	0.842	0.761	0.689
Methapyrilene	No Report	0	0	UD	0	0		0	0
Rotenone	Reported	0.713	0.98	1.05	UD	UD	0.375	20.2	0.128
Staurosporine	No Report	0.685	1.09	1.04	UD	0.612	1.17	0.442	0.837
Tolcapone	No Report	1.23	0.786	0.877	0	0	0.684	0.904	0.826
Trovafloxacin	Reported	0	0	2.38	0	0	0	1.2	0.83

Table 3: Fold-shift calculated according to AC_{50} values for each respective cell health parameter. Undetermined (UD) references when an MEC was not reported for one the ABT treatment conditions. Compounds which did not generate an AC_{50} value in either ABT treatment conditions recorded a fold-shift of 0. A 1.25 cut-off was applied to facilitate toxic metabolite identification (green).

- HCI specific endpoints also showed concordance with the compound specific mechanisms of toxicity. Aflatoxin B1 exo-8,9-epox (AFBO) binds to DNA, resulting in adducts and cytotoxicity. The parameter demonstrating the greatest fold-shift between both ABT conditions was DNA structure (Figure 2)

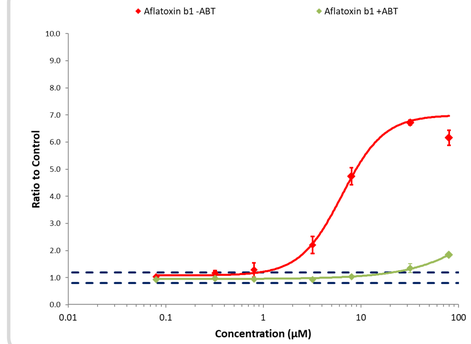

Figure 2: DNA damage HCI cell health feature of HepaRG cell model after aflatoxin B1 treatment in both + and –ABT condition. DNA damage response presents a MEC fold-shift of 23.153 and an AC_{50} of 64.2

Discussion

- The cytotoxicity shifts reported amongst the HepaRG, PHH and PRH cell models dosed with aflatoxin B1 and methapyrilene co-treated alongside ABT, demonstrate the utility of this approach for the identification of toxic metabolites
- Methapyrilene produces a species-specific toxic metabolite that was identified within the rat model but showed no adverse effects on viability within the HepaRG model (Figure 1C 1D), further supporting this assays use in compound risk assessment
- Incorporation of a multiparametric HCI methodology into the existing assay supports compound risk assessment through calculating fold-shift responses according to both MEC and AC_{50} values across all measured cell health parameters (Table 2 & Table 3)
- Through the application of threshold values to such fold-shift calculations novel entities can be flagged for potential risk

Conclusions

- The CYP450 family of enzymes are fundamental in the clearance of various compounds from the body
- Many novel and marketed drugs have been shown to form reactive metabolites through CYP450 mediated pathways, referred to as bioactivation. There is a need to support the identification of such compounds in the early drug discovery process.
- Through application of ABT in the dosing procedure compounds with known toxic metabolite formation can be identified in multiple cell models
- We presented a multiparametric HCI assay which can detect compounds with known reactive metabolites through the HepaRG cell model utilising MEC values