A high content screening approach to genotoxicity testing: detection of DNA damage and differentiation of clastogens and aneugens utilising histone biomarkers.

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Introduction

- Genotoxicity is a leading cause of attrition in drug discovery due to posing a potential carcinogenic hazard.
- Early stage screening *in vitro* is key to avoiding late stage failures.
- Two main classes of genotoxic agents are of concern:
  - Clastogens - which directly damage DNA, for example by intercalating DNA
  - Aneugens - which cause numerical chromosome aberrations, i.e. “lagging chromosomes”
- The nucleosome core protein histone H2A (γH2A.X) is phosphorylated in response to double strand DNA breaks. It is a classic marker for DNA damage.
- Phospho-histone 3 (pH3) is a marker of mitosis. It is upregulated in cells arrested in G2/M and is associated with aneugenicity (1).
- Khuory *et al*., (2016) demonstrated differentiation of aneugens and clastogens based on γH2A.X and pH3 status using the in-cell western technique (2).
- Here we present a modified high content screening (HCS) alternative to the in-cell western protocol incorporating S9 fraction for metabolic activation.
- Compounds are classed by their effect on both γH2A.X and pH3:
  - Clastogens increase γH2A.X only
  - Aneugens increase pH3 expression with either no effect or and increase on γH2A.X
  - Cytotoxic compounds have no effect on either marker

High content imaging of pH3 and γH2A.X staining

- High content screening allows simultaneous quantification of multiparametric indicators of cellular health and biomarkers coupled with automated image analysis.
- HepG2 cells were selected for this study as they have wild type p53, which has been shown to be important in accurate genotoxicity prediction (2)
- HepG2 cells were incubated with compound in the presence and absence of S9 fraction for 24 hours.
- Monolayers were then fixed and stained for pH3 and γH2A.X using standard immunocytochemical techniques.
- Bio-marker levels and cell health markers were quantified using an ArrayScan™ VTI HCS reader (Figure 1A).
- Colchicine, an aneugen, demonstrated a dose dependent increase in pH3 staining. The clastogen etoposide shows an increase in γH2A.X in a dose dependent fashion (Figure 1B).

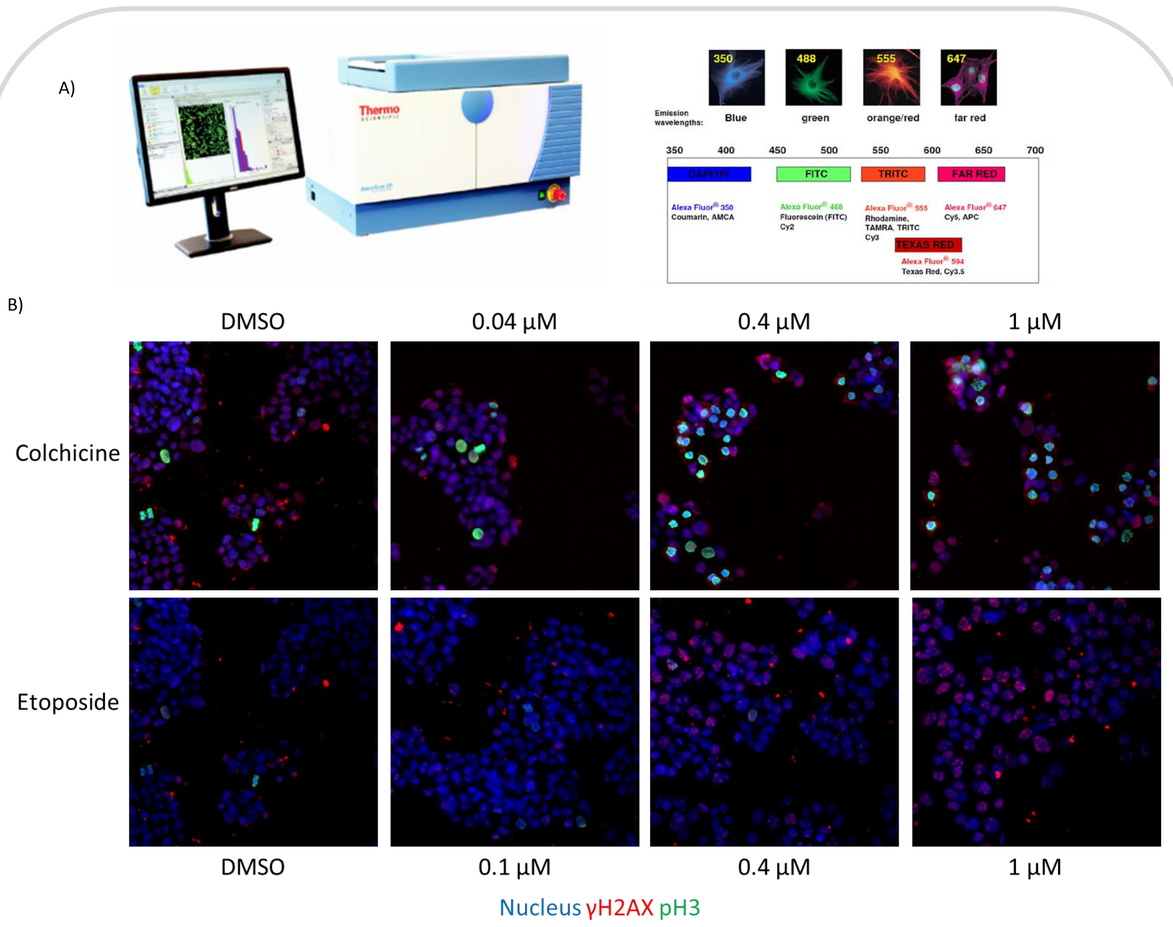

Figure 1: Representative HCS analysis of pH3 and γH2A.X
A) High content screening allows simultaneous quantification of multiple biomarkers. B) Cells were treated with colchicine (aneugen) or etoposide (clastogen) for 24 hours prior to staining for pH3 (green) and γH2A.X (red).

HCS analysis of clastogens and aneugens

- Following automated image analysis compound concentrations with greater than 50% cell loss were excluded at all end points.
- The threshold for a positive response was set at greater than 1.5 fold increase (max response) compared to vehicle only controls.
- Figure 2 shows representative data from the three classes of compounds tested in the absence of S9 fraction.
- Cytotoxic but non-genotoxic chlorpromazine shows no increase in either marker at non-cytotoxic levels. The clastogen etoposide demonstrated an increase in γH2A.X, above the 1.5 fold threshold. Colchicine, an aneugen exhibited a strong response in pH3 while the γH2A.X response showed 1.48 fold max response (table 1B) therefore is below the positive threshold

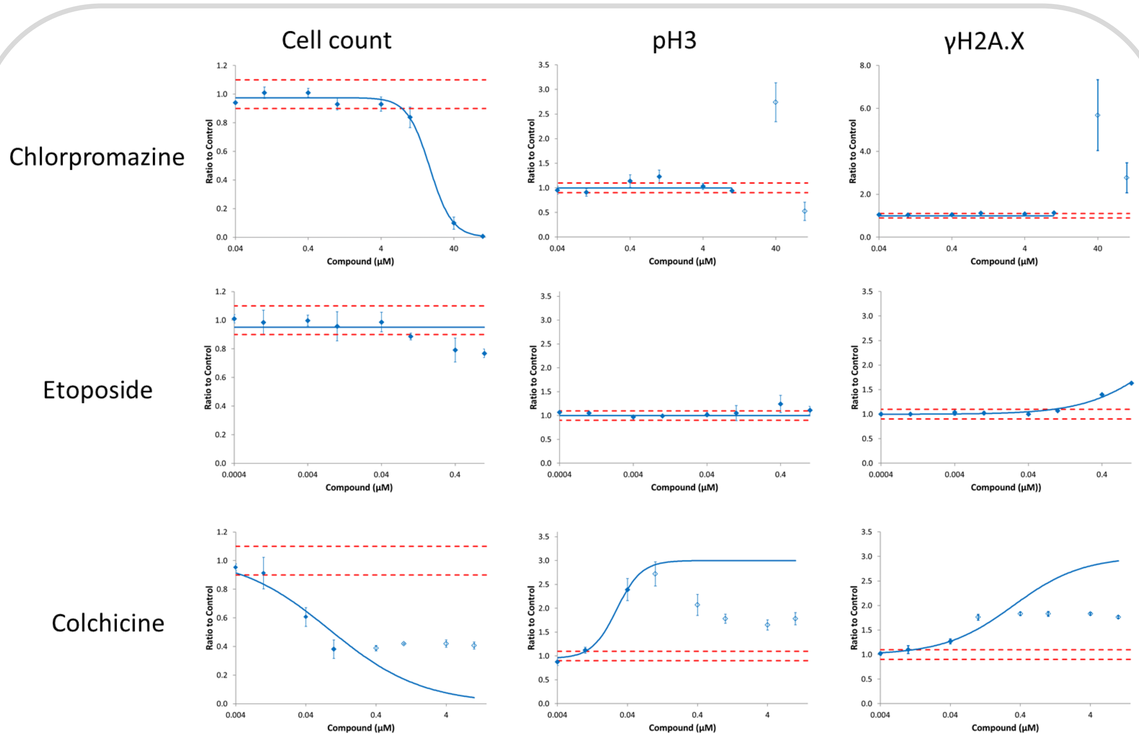

Figure 2: graphical representation of HCS data
HepG2 cells were treated with compound for 24 hours prior to staining for pH3 and γH2A.X. Automated image analysis was performed and concentrations with over 50 % reduction in cell count were excluded for all end-points (open diamonds). Red dashed lines represent vehicle only control levels

Reference compound analysis

- The assay was evaluated using 15 reference compounds in HepG2 cells with a 24 hour incubation time in the presence or absence of 0.5% S9 fraction
- Classification criteria based on pH3 and γH2A.X responses were defined (Table 1 A)
- Cytotoxic compounds were correctly identified in all conditions
- Clastogenic compounds were also correctly identified in all conditions; note cyclophosphamide is metabolically activated therefore is correctly identified as a negative in the –S9 conditions
- Aneugens show an increase in pH3 in –S9 condition but no increase in γH2A.X. These were classed as “cell cycle inhibitors/potential aneugen” and further investigation would be required to conclusively determine their genotoxicity potential. Of the five aneugens tested in +S9 conditions one was classed as “cell cycle inhibitors/potential aneugen”, one classified incorrectly as a clastogen and three correctly identified as aneugens.

Summary

- Histone biomarker responses provided excellent classification of both clastogens and cytotoxic compounds.
- Aneugenic compounds were classed as either “aneugen” or “cell cycle inhibitors/potential aneugen”. This second category may require further investigation, for example using the gold standard *in vitro* micronucleus test.
- This HCS assay is a useful addition to genotoxicity screening, allowing differentiation of classes of genotoxic compounds and identification of non-genotoxic cytotoxic compounds.

References:
1) Muehlbauer and Schuler (2005) Mut Res pp156-169 DOI:10.1016/j.mrgentox.2005.05.002; 2) Khoury *et al* (2016) Arch Toxicol 90(8) pp1983-1995; DOI:10.1007/s00204-015-1599-1; 3) Kumari *et al*. (2014) Mol Cell Oncol 1(3) DOI: 10.4161/23723548.2014.969653

A)

+ pH2aX	- PH3	=	Clastogen
	+ PH3	=	Aneugen
- pH2aX	- PH3	=	Cytotoxic/- ve
	+ PH3	=	Cell cycle inhibitor/Aneugen

Threshold 1.5

Table 1: summary of screened compounds:
HepG2 monolayers were treated for 24 hours with and without S9 fraction. Any data point where over 50 % cell loss had occurred were excluded. Mechanism were predicted with criteria set in A), dark green boxes show correctly predicted compounds. B) The positive threshold was set at 1.5 fold increase compared to vehicle only controls. Orange boxes are above the threshold, pale green boxes are below the threshold. * Cyclophosphamide is metabolically activated.

B)

		-S9				+S9				Mechanism	
		pH2aX		pH3		pH2aX		pH3			
Compound	Mechanism	MEC	Max response	MEC	Max response	MEC	Max response	MEC	Max response	-S9	+S9
vinblastine	Aneugen	NR	NR	<0.004	2.28	0.0193	1.6	<0.004	2	Cell cycle inh/Aneugen	Aneugen
colchicine		0.022	1.47	0.0112	4.2	0.0592	1.39	0.00889	1.93	Cell cycle inh/Aneugen	Cell cycle inh/Aneugen
paclitaxel		0.325	1.3	0.006	3.91	0.189	1.41	0.0253	3.95	Cell cycle inh/Aneugen	Aneugen
carbendazim		NR	NR	1.56	2.74	0.0272	3.23	0.0136	1.34	Cell cycle inh/Aneugen	Clastogen
griseofulvin		17.2	1.41	4.54	3.2	26.1	1.74	8.81	2.68	Cell cycle inh/Aneugen	Aneugen
methyl methanesulfonate	Clastogen	124	2.53	NR	NR	41.1	3.49	264	1.34	Clastogen	Clastogen
etoposide		0.141	1.63	NR	NR	0.288	1.61	NR	NR	Clastogen	Clastogen
4-nitroquinoline N-oxide		0.277	6.18	1.76	-0.553	0.727	6.97	NR	NR	Clastogen	Clastogen
chlorambucil		2.12	3.05	NR	NR	4.15	7.01	185	1.31	Clastogen	Clastogen
cyclophosphamide		NR	NR	NR	NR	17.6	1.86	166	1.2	Cytotoxic/- ve*	Clastogen
araC		0.029	3.95	NR	NR	0.0272	3.23	0.0245	1.32	Clastogen	Clastogen
7, 12-dimethylbenz[a]anthra		<0.08	1.57	NR	NR	1.55	1.81	NR	NR	Clastogen	Clastogen
chlorpromazine	Cytotoxic	NR	NR	NR	NR	NR	NR	NR	NR	Cytotoxic/- ve	Cytotoxic/- ve
CCCP		1.22	1.43	NR	NR	6.66	1.3	7.01	1.33	Cytotoxic/- ve	Cytotoxic/- ve
starousporine		0.179	1.27	<0.012	-0.257	2.73	1.27	0.203	-0.655	Cytotoxic/- ve	Cytotoxic/- ve

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