High Throughput Genotoxicity Profiling of the US EPA ToxCast™ Chemical Library

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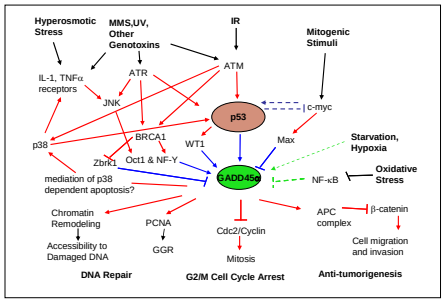
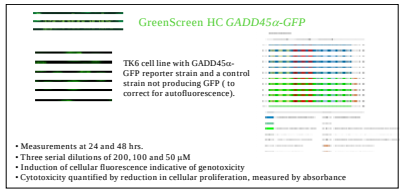
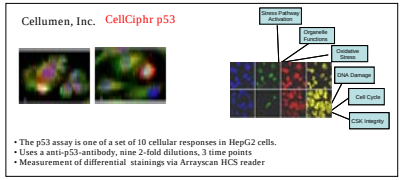
INTRODUCTION

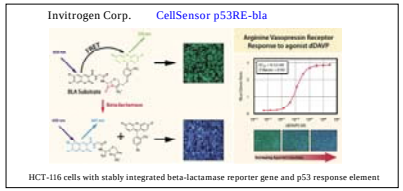
A key aim of the ToxCast™ project[1] is to investigate the use of modern high-throughput screening (HTS) assays to provide a biologically informed basis for predicting toxicity and prioritizing chemicals for further testing. The purpose of this study is to evaluate recently developed HTS methodologies from Gentronix Ltd., Cellumen Inc., and Invitrogen Corp., the latter performed by the National Institutes of Health Chemical Genomics Center (NCGC), in the context of the particular chemical space and larger aims of the ToxCast™ project. These assays each indicate different aspects of the cellular response to a genotoxic challenge, which can lead to DNA damage, mis-repair and mutations and, ultimately, to tumorigenesis and carcinogenesis. The Gentronix 'GreenScreen HC' assay[2] uses a human lymphoblastoid TK6 cell line genetically modified to include a green fluorescent protein (GFP) reporter for the human GADD45a gene. The Cellumen 'CellCiphr' cytotoxicity profile panel[3] includes fluorescent probes for 10 cellular responses including DNA damage in human HepG2 cells as measured by p53 activation using fluorescent anti-p53 antibody. The Invitrogen 'CellSensor' assay[4] uses a beta-lactamase reporter gene under the control of a p53 response elements stable integrated in HCT-116 cells and measured via fluorescent resonance energy transfer (FRET). These HTS assays represent two gene targets in their endpoints, p53 and GADD45a in a p53 competent cell line. P53 is known to act as a 'gate keeper' to ensure genetic and cellular integrity during the cell cycle. GADD45a (growth arrest and DNA damage) mediates the cell's response to genotoxic stress. The HTS assay results were combined with the ToxCast™ Phase I compound data set. This set consists of 320 primarily pesticidal active compounds for which hazard assessment toxicological data, including multisite tumorigenicity data, are available from the US EPA Office of Pesticide Programs. HTS assay results are also compared with historical mutagenicity (Ames) data available for a subset of the test compounds. The purpose of these HTS assays is not to replace the use of Ames tests, but to increase testing efficiency for early screening of larger sets of chemicals of interest.



METHODS







HTS Methods Comparison

	GreenScreen HC	CellCiphr p53	CellSensor p53
Endpoints	Genotoxicity / Cytotoxicity	Genotoxicity / Cytotoxicity	Genotoxicity
Microplate	96 well	384 well	1536 well
Number of compounds / plate	12	16	1408
Number of dilutions / compound	3	10	12 (in 12 microplates)
Incubation time / hr	24 and 48	24 to 72	24
Compounds per week (typical)	720	100 - 500	30,000 - 100,000
Equipment required	Conventional microplate reader (Tecan Infinite F500)	Image analysis equipment (ArrayScan)	Microlitre liquid handling automation on ultra-high throughput robotic platforms
Data interpretation	Straightforward and automated - using proprietary Excel software template	Specialist image analysis software - 1 of 10 endpoints therefore data extraction required	Straightforward and automated - using proprietary software. Some user input to identify
Reagents	Proprietary cell lines and media from Gentronix	Proprietary cell lines and media from Millipore	Proprietary cell lines and media from Invitrogen

Replicate Sample Reproducibility

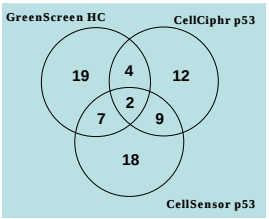
(for replicate compounds randomly distributed in the compound library)

ChemicalName	CAS Number	Replicate #	Source	Purity (%)	GreenScreen HC Genotoxicity	GreenScreen HC Cytotoxicity	CellCiphr p53 Genotoxicity	CellCiphr p53 Cytotoxicity
3-Iodo-2-propynylbutylcarbamate	55406-53-6	1	Crescent	97	+ +	+ +	+	+
		2	Sigma	97.1	+ +	+ +	+	+
Bensulide	741-58-2	1	Sigma	99.5	-	+ +	-	+
		2	Sigma	99.5	-	+ +	-	+
		3	Sigma	99.5	-	+ +	-	+
Chlorsulfuron	64902-72-3	1	Crescent	99	-	-	-	-
		2	Sigma	99.9	-	-	-	-
Dibutyl phthalate	84-74-2	1	Sigma	99.63	-	+ +	-	+
		2	Alfa_Aesar	98.6	-	+	-	+
Diclofop-methyl	51338-27-3	1	Sigma	99.2	-	+ +	-	-
		2	Sigma	99.2	-	+ +	-	+
		3	Sigma	99.2	-	+ +	-	+
EPTC	759-94-4	1	Sigma	98.7	-	-	-	-
		2	Crescent	97	-	-	-	-
Fenoxaprop-ethyl	66441-23-4	1	Crescent	98	-	+ +	-	+
		2	Sigma	98.4	-	+ +	-	+
Prosulfuron	94125-34-5	1	Sigma	98.4	-	-	-	-
		2	Sigma	98.4	-	+	-	-
		3	Sigma	98.4	-	-	-	-

Summary of HTS predicted positive assay data

Assay	Genotoxicity		Cytotoxicity	
	Number	%	Number	%
GreenScreen HC	32	10.4	231	74.8
CellCiphr p53	27	8.7	171	55.3
CellSensor p53	36	11.7	-	-

Overlap of HTS predicted positive genotoxicity assay data



Concordance with Multiple Site Rodent Tumorigenicity Data

	GreenScreen +	GreenScreen -
Rodent +	13	45
Rodent -	16	199

	CellSensor +	CellSensor -
Rodent +	10	48
Rodent -	23	192

	Cellciphr +	Cellciphr -
Rodent +	8	50
Rodent -	18	197

	Ames +	Ames -
Rodent +	14	14
Rodent -	22	40

	GreenScreen	CellSensor	CellCiphr	Ames
Number of comparisons	273	273	273	90
Sensitivity (% correct positives)	22.4	17.2	13.8	50.0
Specificity (% correct negatives)	92.6	89.3	91.6	64.5
Concordance	77.7	74.0	75.1	60.0
Balanced Accuracy	57.5	53.3	52.7	57.3
Relative Predicitivity (Positives)	3.01	1.61	1.65	1.41
Relative Predictivity (Negatives)	1.19	1.08	1.06	1.29

CONCLUSIONS

Assay Statistics The number of positive results for genotoxicity was similar between the 3 assays, averaging just over 10%. Whereas the GreenScreen HC, CellCiphr p53 and CellSensor p53 assays produced similar numbers of positive results (32, 27 and 36 respectively), the overlap between data sets was reasonably small. The number of positive GreenScreen HC results that were common to CellCiphr p53 and CellSensor p53 assays were 6 and 9, respectively. There were 11 CellCipher p53 positive assay results that were common to CellSensor p53. The historical Ames test data had 46 positive results for 108 tested compounds.

Correlation with rodent bioassay The HTS assays have a lower number of true positive results compared to the Ames assay due to lower sensitivity, a tradeoff for the advantage of screening large numbers of compounds with low sample concentration requirements. The Ames assay predicts a greater percentage of true positives (higher sensitivity), but it also produces a larger percentage of false positives (or smaller percentage of correct negatives), i.e. lower specificity. Conversely the HTS assays demonstrated high specificity, consistently over 88%, and hence produce a low number of what may be construed as false positive results.

Overall strategic learning The present analysis suggests that HTS genotoxicity assays could be used as an *in vitro* screen for potentially genotoxic compounds. As one part of a weight-of-evidence assessment, the data could be applied to judge the likelihood of a compound's potential adverse effect for humans, to help determine the mode of action for carcinogenicity, to enhance the process of prioritization by selecting compounds for further study.

REFERENCES

(1) EPA ToxCast, 2009. U.S. Environmental Protection Agency's National Center for Computational Toxicology ToxCast Project, http://www.epa.gov/ncct/toxcast; Accessed January 2009.
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(4) Invitrogen, 2009. CellSensor™ p53RE-bla HCT-116 Cell-based Assay Protocol. http://tools.invitrogen.com/content/sfs/manuals/cellsensor_p53HCT116_man.pdf, Accessed January 2009.

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