In Vitro Micronucleus and CometChip® with Metabolically Competent HepaRG™ Cells

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Abstract

Human hepatoma-derived HepaRG™ cells retain many of the metabolic capabilities of primary human hepatocytes and represent a readily available model of metabolically-competent cells for use in genetic toxicity testing. Integrated Laboratory Systems (ILS) is incorporating HepaRG™ cells into medium-throughput micronucleus and comet assay formats. Use of HepaRG™ cells offers flexibility in dose scheduling and assay design since the need for addition of exogenous S9 is eliminated. In collaboration with researchers at MIT and Health Canada, ILS has successfully demonstrated the ability of HepaRG™ cells to detect genetic damage. HepaRG™ cells seeded into collagen-coated plates were allowed to recover metabolic functions for 7 days, then exposed to test compounds daily for 3 days. Using Litron's *In Vitro* Microflow® kit, chromosomal damage was detected with 100% specificity and 100% sensitivity following exposure to 6 non-genotoxins (e.g. phenobarbital, 2-deoxy-D-glucose) and 8 known genotoxins, including 1 non-DNA reactive genotoxin (colchicine) and 3 genotoxins requiring metabolic activation (cyclophosphamide, aflatoxin B1, benzo[a]pyrene). Exposure to ethylmethane sulfonate, methylmethane sulfonate, cyclophosphamide, and benzo[a]pyrene induced similar response patterns in a side-by-side comparison of the CometChip® (Trevigen®) and the comet assay using standard 2-well Trevigen® slides. In addition, expression analysis of several metabolism-related genes evaluated in cyclophosphamide-exposed HepaRG™ cells demonstrated dose-related increases in expression of *CYP2B6*, *CYP3A4*, and *UGT1A1*, indicating appropriate metabolic functionality of the exposed cells. A panel of 60+ chemicals covering a range of genotoxic activities and mechanisms has been evaluated in the CometChip® format. Responses have been consistent with expected outcomes and with published literature, and correlate well with concurrent cytotoxicity evaluation (ATP quantitation, CellTiter-Glo®, Promega). Assays incorporating HepaRG™ cells can provide potential follow up to positive results from S9-based *in vitro* assays used for regulatory testing.

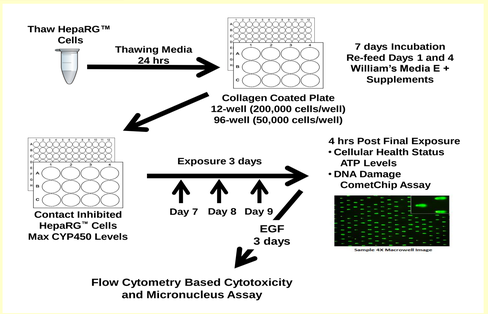
Introduction

- The *in vitro* micronucleus (MN) assay is a well-established test for cytogenetic damage in mammalian cells and is accepted as a regulatory genetic toxicity assay by the ICH. The current OECD test guideline for this assay (#487) has been recently revised.
- The comet assay for DNA strand breaks is a well-established test for tissue-based DNA damage and an OECD guideline for the in vivo comet assay (#489) has been recently established.
- As part of Health Canada's effort to establish a gene signature for detecting potential exposure to genotoxins, ILS has tested a number of compounds in the *in vitro* micronucleus assay to provide a phenotypic anchor for genotoxic versus non-genotoxic response[1].
- In collaboration with the Massachusetts Institute of Technology, ILS is developing a medium-throughput, *in vitro* version of the comet assay using the CometChip® technology[2].
- HepaRG™ cells offer a convenient metabolically-competent test system for use in genetic toxicity assays. ILS has adapted both the *In Vitro* MicroFlow® assay[3] and the CometChip® assay[2] for use with HepaRG™ cells.

HepaRG™ Cells

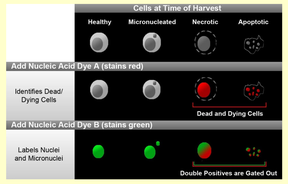
- Isolated from a hepatocarcinoma from a female patient[4]. When differentiated from a low density culture, both hepatocytes and biliary cells are formed, creating a natural co-culture system.
- Differentiated HepaRG™ cells retain activities of a number of metabolism enzymes and receptors: CYP1A1, CYP2B6, CYP2C9, CYP2E1, CYP3A4, PXR, CAR, PPARα, AhR, GSTA1, GSTA4, GSTM1, UGT1A1.

Treatment Procedure



Methods: *In Vitro* MicroFlow® Micronucleus Assay and CometChip®

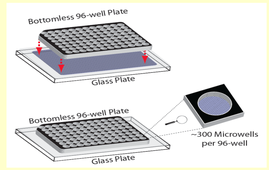
In Vitro MicroFlow® Micronucleus Assay



- Relative Survival
- Apoptosis/Necrosis
- Cell Cycle Arrest
- Micronucleus Frequency

Figure 1

CometChip® Assay for DNA Damage



A bed of microwells in an agarose gel is cast and then compressed under a bottomless 96-well plate to create the CometChip®.

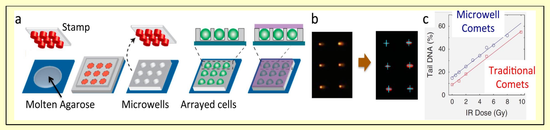
Figure 2

Advantages of the CometChip® Technology

Advantages of CometChip® over traditional comet:

- **Analysis of arrayed cells prevents overlap**
- **Parallel processing of 96 samples suppresses sample-to-sample variation**
- **Shared focal plane enables multiple comets per image**
- **Automated image analysis program allows rapid data collection**
- **The physics of the microwell results in reduced comet-to-comet variation**
- **More than an order of magnitude fewer images required per condition**

CometChip® Assay for DNA Damage



a) Microfabricated mold creates precision microwells that can be loaded with single or groups of cells.
b) Software identification of arrayed comets enables automated analysis.
c) Validation of CometChip® data.

Figure 3

HepaRG™ Cells Are a Robust Test System for Micronucleus Detection

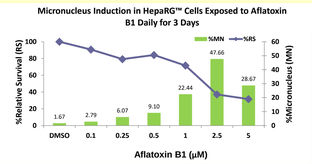


Figure 4

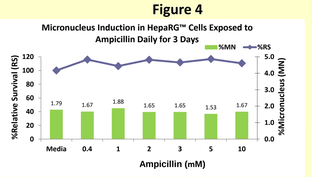
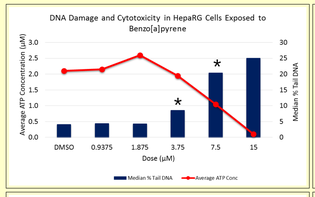


Figure 5

Test Chemical	Predicted Response	Result
Aflatoxin B1	+	+
Benzo[a]pyrene	+	+
Cisplatin	+	+
Colchicine	+	+
Cyclophosphamide	+	+
Etoposide	+	+
Methyl methanesulfonate	+	+
2-Nitrofluorene	+	+
Ampicillin	–	–
Ascorbate (sodium salt)	–	–
2-Deoxy-d-glucose	–	–
Phenobarbital	–	–
Sodium chloride	–	–
Sucrose	–	–

Screening of Recommended Chemicals[5] for DNA Damage Using the CometChip® and HepaRG™ Cells

Known Genotoxins



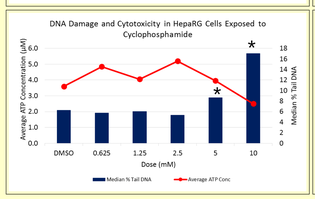
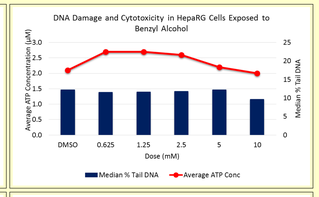


Figure 6

Suspected Non-Genotoxins



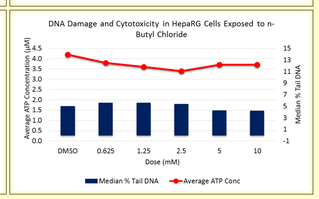
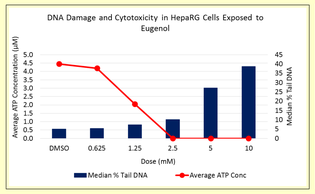


Figure 7

Chemicals of Uncertain Genotoxicity



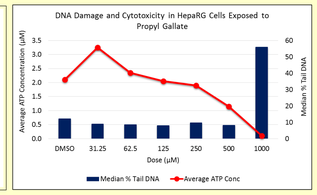
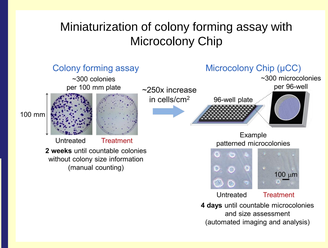
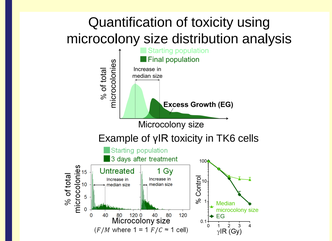
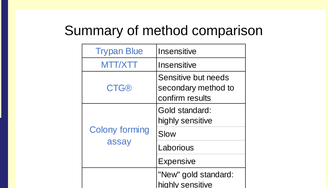


Figure 8

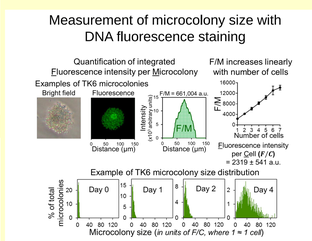
Development of Microcolony Chip® for Assessment of Cytotoxicity







Trypan Blue	Insensitive
MTT/XTT	Insensitive
CTG®	Sensitive but needs secondary method to confirm results
Colony forming assay	Gold standard: highly sensitive
	Slow
	Laborious
	Expensive
Microcolony Chip	"New" gold standard: highly sensitive
	Fast
	Automatic
	Inexpensive



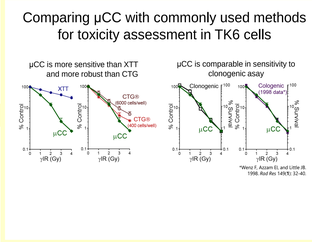


Figure 9

Screening of Recommended Chemicals[5] for DNA Damage Using the CometChip® and HepaRG™ Cells

In-vivo genotoxins	Maximum Dose Required by OECD for Neg Call w/o Cytotoxicity	Maximum Dose Tested	Cytotoxicity	Doses to Achieve Stated Cytotoxicity(ies)	Final Result
I. Ames-positive in-vivo genotoxins					
O6 and N7 alkylators					
Cyclophosphamide (6055-19-2)	2 mg/mL	10 mM (2.79 mg/mL)	31%	10 mM	Positive
ENU (759-73-9)	10 mM	5 mM	15%; 75.5%	2.5 mM; 5 mM	(Positive)
MMS (66-27-3)	10 mM	40 µg/mL (0.36 mM)	34%	40 µg/mL	Positive
Polycyclic aromatic hydrocarbons					
Benzo[a]pyrene (50-32-8)	2 mg/mL	15 µM (0.0038 mg/mL)	50%; 95%	7.5 µM; 15 µM	Positive
7,12-Dimethylbenzanthracene (57-97-6)	2 mg/mL	1.17 mM (0.3 mg/mL)	34.7%	1.17 mM	(Positive)
Aromatic amines					
Dimethylnitrosamine (62-75-9)	10 mM	10 mM	19.8 %; 30.8%	10 mM (DMSO; water)	(Negative)
2-Acetylaminofluorene (53-96-3)	2 mg/mL	10 mM (2.23 mg/mL)	51.4%	10 mM	(Negative)
2,4-Diaminotoluene (95-80-7)	10 mM	10 mM	30.9%; 57.4%	5 mM; 10 mM	(Negative)
Others					
Aflatoxin B1 (1162-65-8)	2 mg/mL	50 µM (0.016 mg/mL)	29.1%; 69.6%	12.5 µM; 25 µM	(Positive)
Cadmium chloride (10108-64-2)	10 mM	1000 µM	53.3%	15 µM	(Negative)
Cisplatin (15663-27-1)	2 mg/mL	50 µM (0.151 mg/mL)			(Positive)
p-Chloroaniline (106-47-8)	10 mM	10 mM	57.7%	2.5 mM	(Negative)
II. In-vivo genotoxins negative or equivocal in Ames					
Etoposide (33419-42-0)	2 mg/mL	50 µM (0.029 mg/mL)			
Hydroquinone (123-31-9)	10 mM	10 mM	25%; 100%	750 µM; 1000 µM	(Negative)
Azidothymidine (30516-87-1)	2 mg/mL	7.5 mM (2 mg/mL)	11.1%	7.5 mM	(Negative)
Sodium arsenite (7784-46-5)	10 mM	1000 µM	88.4%	62.5 µM	(Negative)
Taxol (33069-62-4)	2 mg/mL	1000 µM (0.85 mg/mL)	56%	100 µM	(Negative)
Chloramphenicol (56-75-7)	2 mg/mL	2 mg/mL	58.3%	1 mg/mL	(Negative)
Non-DNA reactive chemicals					
non-carcinogens with negative in-vivo genotoxicity data					
Ampicillin trihydrate (7177-48-2)	2 mg/mL	5 mM (2 mg/mL)	None	NA	(Negative)
D-Mannitol (69-65-8) –ve [3]	10 mM	10 mM	None	NA	(Negative)
non-carcinogens with no in-vivo genotoxicity data					
Phenformin HCl (834-28-6)	2 mg/mL	200 µM (0.048 mg/mL)	47%; 70%	100 µM; 200 µM	(Negative)
n-Butyl chloride (109-69-3)	10 mM	10 mM	None	NA	(Negative)
Chlorocholine (999-81-5)	10 mM	10 mM	17.1%	10 mM	(Negative)
Cyclohexanone (108-94-1)	10 mM	10 mM	None	NA	(Negative)
N,N-Dicyclohexyl thiourea (1212-29-9)	2 mg/mL	2 mM (0.48 mg/mL)	42%	2 mM	(Negative)
Erythromycin stearate (643-22-1)	2 mg/mL	2 mM (~2 mg/mL)	56.1%	100 µM	(Negative)
Fluorneturon (2164-17-2)	2 mg/mL	5 mM (1.16 mg/mL)	60.7%	1 mM	(Negative)
Phenanthrene (85-01-8)	10 mM	10 mM	42.6%	10 mM	(Negative)
non-genotoxic carcinogens					
D-Limonene (5989-27-5)	10 mM	10 mM	66.6 %; 100 %	1.25 mM; 2.5 mM	(Negative)
Di-(2-Ethylhexyl)phthalate (117-81-7)	2 mg/mL	2 mg/mL	46.9%; 79.7%	0.25; 0.5 mg/mL	(Negative)
Amitrole (61-82-5)	10 mM	10 mM	None	NA	(Negative)
tert-Butyl alcohol (75-65-0)	10 mM	10 mM	None	NA	(Negative)
Diethanolamine (111-42-2)	10 mM	10 mM	53.1%	5 mM	(Negative)
Melamine (108-78-1)	1 mM	1 mM	(NA)	None	(Negative)
Progesterone (57-83-0)	2 mg/mL	119 µM (0.037 mg/mL)	46.7 %; 100 %	119 µM; 238.5 µM	(Negative)
Pyridine (110-86-1)	10 mM	10 mM	None	NA	(Negative)
Tris(2-ethylhexyl)phosphate (78-42-2)	2 mg/mL	10 mM (4.34 mg/mL)	38%	0.625-2.5 mM	(Negative)
Hexachloroethane (67-72-1)	2 mg/mL	7.5 mM (1.78 mg/mL)	44.2%	250 µM	(Negative)
Non-DNA reactive chemicals that test positive at high concentrations or high levels of cytotoxicity					
Non-carcinogens that are negative or equivocal for genotoxicity in vivo					
d,l-Menthol (15356-70-4)	10 mM	10 mM	24 %; 100 %	1.25 mM; 2.5 mM	(Negative)
Phthalic anhydride (85-44-9)	10 mM	10 mM	16.7 %; 100 %	5 mM; 10 mM	(Negative)
tert-Butylhydroquinone (1948-33-0)	10 mM	2 mM	55.6%	500 µM	(Negative)
o-Anthranilic acid (118-92-3)	10 mM	10 mM	50%	10 mM	(Negative)
1,3-Dihydroxybenzene (resorcinol) (108-46-3)	10 mM	10 mM	20.7 %; 100 %	5 mM; 10 mM	Positive
2-Ethyl-1,3-hexanediol (94-96-2)	10 mM	10 mM	38%	10 mM	(Negative)
Sulfloxazole (127-69-5)	2 mg/mL	10 mM (2.67 mg/mL)	43.2%; 63.5%	2.5 and 5 mM; 10 mM	Negative
Non-carcinogens with no in vivo genotoxicity data					
Ethionamide (536-33-4)	10 mM	10 mM	minimal; 82.7 %	2.5 mM; 5 mM	Negative
Curcumin (458-37-7)	2 mg/mL	100 µM (0.037 mg/mL)	20-40%; 100%	25-50 µM; 100 µM	(Negative)
Benzyl alcohol (100-51-6)	10 mM	10 mM	None	NA	Negative
Urea (57-13-6)	10 mM	10 mM	None	NA	(Negative)
Non-genotoxic carcinogens or carcinogenic by irrelevant (for humans) mechanism					
Sodium saccharin (128-44-9)	2 mg/mL	10 mM (2.05 mg/mL)	None	NA	(Negative)
Supplementary Chemicals					
Supplementary list					
Propyl gallate (121-79-9)	2 mg/mL	1 mM (0.21 mg/mL)	45%; 100%	500 µM; 1 mM	Negative
p-Nitrophenol (100-02-7)	10 mM	10 mM	none; 100%	312.5; 625 µM	(Negative)
Sodium xylene sulfonate (1300-72-7)	2 mg/mL	10 mM (2.98 mg/mL)	None	NA	(Negative)
Ethyl acrylate (140-88-5)	10 mM	10 mM	None	NA	Negative
Eugenol (97-53-0)	10 mM	10 mM	54%	1.25 mM	Negative
Isobutyraldehyde (78-84-2)	10 mM	10 mM	None	NA	Negative
2,4-Dichlorophenol (120-83-2)	10 mM	1 mM	50%	500 µM	Negative
Additions from OECD guidelines and previous ILS testing					
EMS (62-50-0)	10 mM	8 mM	none; 100%	4 mM; 8 mM	(Positive)
2-Nitrofluorene (607-57-8)	2 mg/mL	400 µM	49.1%; 61%	200 µM; 400 µM	(Negative)
Colchicine (64-86-8)	2 mg/mL	50 µM	None	NA	
2-Deoxy-D-glucose (2-DG)	10 mM	10 mM	None	NA	(Negative)
Sucrose	10 mM	10 mM	None	NA	(Negative)
Sodium chloride	10 mM	10 mM	None	NA	(Negative)
Mitomycin C	2 mg/mL	100 ng/mL	None	NA	
4-Nitro-quinoline-N-oxide	10 mM	5 µg/mL	24.7%; 80.2%	0.625; 1.25 µg/mL	(Positive)
Cytosine arabinoside	2 mg/mL	200 µM	None	NA	(Positive)

CometChip® as a Medium-Throughput Assay for DNA Damage

- Rapid turn-around of comet results
- Multiple chemicals per array for cost savings
- Excellent screening tool; adaptable for regulatory studies
- Adaptable to automation
- Increased flexibility for dosing schedules - no S9 with HepaRG™ cells

References

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The authors would like to acknowledge Kim Shepard, Nick Christy, and Angela Rauer in the Genetic Toxicology Group at ILS, and Kevin Ketner, for their contributions to the work presented.

This work was funded in part through NIH-funded Small Business Innovative Research grant # 4R44ES024698-02 in collaboration with researchers at MIT.

Funding for the *in vitro* micronucleus work was provided through Health Canada's Genomics Research and Development Initiative.