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In vitro **Toxicology**

γH2AX Double Strand DNA Damage Response Assay

Background Information

'Taking into account the ability of the automated γH2AX assay to predict genotoxicity *in vivo* to the same accuracy as currently used *in vitro* assays, while its use of human metabolic competent cells, and its automated scoring, its limited use of test compound since small volumes are needed and its simple and rapid applicability to study large numbers of chemicals as it is amenable to robotised procedures, there are many arguments in favor of its usefulness as *in vitro* genotoxicity test. Especially its high sensitivity to detect DNA-reactive GTX compounds is a positive asset.'

[3]Tsamou *et al*. (2012), *Mutagenesis* **27(6)**; 645-652

- Histone H2A variant H2A.X, a component of the nucleosome core structure has a special role in DNA repair. Phosphorylation of H2A.X at residue Ser-139 (Anti-γH2A.X) by PI3K-like kinases, including ATM, ATR and DNA-PK, is an early cellular response to the generation of DNA double-strand breaks (DSBs)[1].
- Detecting the formation of DSBs using the γH2A.X (Ser-139) has emerged as a highly specific and sensitive molecular marker for monitoring DNA damage[1].
- DSBs form when both strands of the DNA double helix are broken, irrespective of how they are formed they are found to be highly toxic and can ultimately be fatal[2].
- Cyprotex's DNA Damage assay uses High Content Screening (HCS) to identify both DNA Damage and Cytotoxicity.

Protocol

Cell Line
HepG2 (other cell types available on request)

Multiplexing
Combination with other mechanistic endpoints available on request
Compatible with the *in vitro* HCS Micronucleus Test (MNT)

Analysis Platform
Cellomics ArrayScan® VTI or XTI (Thermo Scientific)

Test Article Concentrations
8 point dose response curve with highest concentration based on cell loss or solubility limit (3 replicates per concentration)

Test Article Requirements
50 µL solution at 200x highest concentration or equivalent amount in solid

Time Points
In absence or presence of aroclor 1254 induced rat liver S9: 24 hr exposure time

Quality Controls
Negative control: 0.5% DMSO (vehicle)
Positive controls: Cyclophosphamide (S9 positive control) and chlorambucil (positive control)

Data Delivery
Minimum effective concentration (MEC) and AC_{50} value for each measured parameter (cell loss, nuclear morphology, DNA fragmentation and DNA damage)

'The γH2AX assay is based on the total phosphorylation of H2AX histone in response to DNA damage by induction of double-strand breaks (DSBs).[3]'

Figure 1

Representative HCS images for cells treated with (A) 200μM chlorambucil (positive control) (B) vehicle control in the absence of S9 fraction (C) 200μM cyclophosphamide (metabolising system positive control) and (D) vehicle control in the presence of S9 fraction over a 24 hr period. Cell nuclei are stained blue (Hoechst) with pink staining observed in the nucleus of cells positive for γH2A.X (indicated by white triangles). Cellular filamentous actin is stained green (phalloidin).

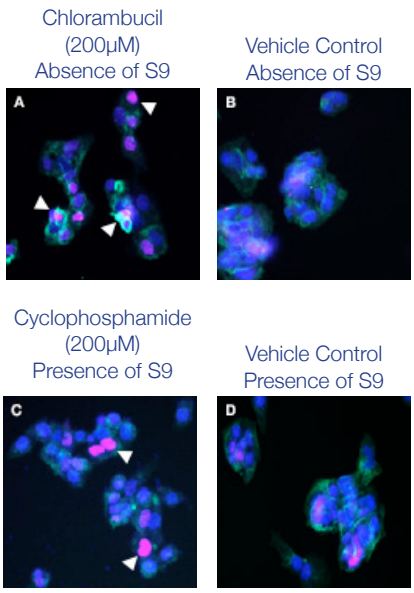


Table 1

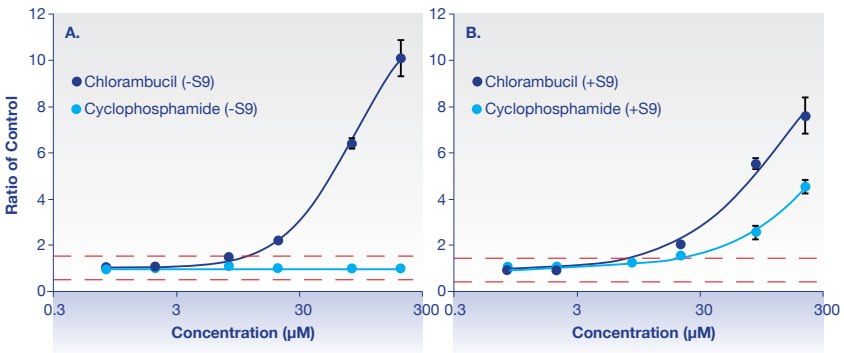
γH2A.X data for 24 validation compounds categorised according to literature data[3-5].

	Minus Rat Liver S9			Plus Rat Liver S9		
Compound	MEC	AC_{50}	+ve/-ve	MEC	AC_{50}	+ve/-ve
In vivo genotoxin						
Benzo(a)pyrene	0.133	29.8	+	1.93	103	+
Chlorambucil	9.69	55.2	+	7.53	66.2	+
Cisplatin	0.356	8.64	+	0.589	11.7	+
Colchicine	0.03	>0.2	+	0.0239	0.194	+
Cyclophosphamide	NR	NR	-	11.3	253	+
Cytarabine	0.0175	0.964	+	0.0376	10.8	+
Etoposide	0.346	5.23	+	0.703	>10	+
Formaldehyde	145	>400	+	97.6	825	+
Griseofulvin	8.56	>300	+	12.5	>300	+
Hydroxyurea	274	442	+	301	506	+
Mitomycin C	0.0116	1.12	+	0.0788	2.35	+
Vinblastine	0.0054	>0.02	+	0.0091	>0.05	+
In vivo non-genotoxin						
Cyclosporin A	NR	NR	-	NR	NR	-
Diclofenac	NR	NR	-	NR	NR	-
Acrylonitrile	NR	NR	-	NR	NR	-
Amoxicillin	NR	NR	-	NR	NR	-
Cefuroxime	NR	NR	-	NS	NS	-
Hydrocortisone	NR	NR	-	NR	NR	-
Lansoprazole	NR	NR	-	NR	NR	-
Nalidixic Acid	NR	NR	-	NR	NR	-
Citalopram	NR	NR	-	NR	NR	-
Eugenol	NR	NR	-	NR	NR	-
Norfloxacin	NR	NR	-	NR	NR	-
Resorcinol	NR	NR	-	NR	NR	-

NR = no response NS = not significant

Figure 2

Graphical representation of γH2A.X for chlorambucil (positive control) and cyclophosphamide (positive control for metabolising system). A: is in the absence of aroclor 1254 induced rat liver S9. B: is in the presence of aroclor 1254 induced rat liver S9. Red dashed line represents the vehicle control limits.



Chlorambucil causes a concentration dependent increase in γH2A.X compared to vehicle control treated cells in both the absence and the presence of metabolising system (aroclor 1254 induced rat liver S9). No response was observed for cyclophosphamide in the absence of metabolising system, however in the presence of the metabolising system cyclophosphamide shows a concentration dependent increase in γH2A.X. Data represents mean of triplicate incubations ± standard deviation.

HepG2 cells were treated for 24 hours with test compound in the absence and presence of aroclor 1254 induced rat liver S9. The compounds were analysed using Cellomics ArrayScan® VTI or XTI (Thermo Scientific). Cyprotex data correlates well with literature *in vivo* data[3-5].

References

[1] Mah LJ *et al.*, (2010) γH2A.X: a sensitive molecular marker of DNA damage and repair. *Leukemia* **24**; 679 – 686
[2] Chapman JR *et al.*, (2012) Playing the End Game: DNA Double-Strand Break Repair Pathway Choice. *Molecular Cell* **47(4)**; 497 – 510
[3] Tsamou M *et al.*, (2012) Performance of *in vitro* γH2A.X assay in HepG2 cells to predict *in vivo* genotoxicity. *Mutagenesis* **27(6)**; 645-652
[4] Diaz D *et al.*, (2007) Evaluation of an automated *in vitro* micronucleus assay in CHO-K1 cells. *Mutation Research* **630**; 1-13
[5] Hastwell PW *et al.*, (2009) Analysis of 75 marketed pharmaceuticals using the GADD45a-GFP 'GreenScreen HC' genotoxicity assay. *Mutagenesis*, **24(5)**; 455–463