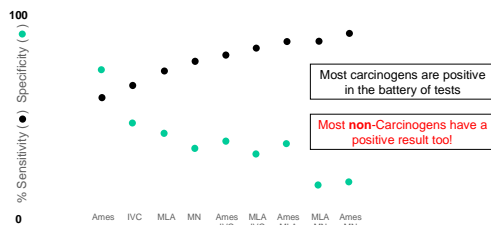


Introduction.

The regulatory *in vitro* mammalian genotoxicity tests have poor specificity leading to many confounding false positive results. Here we describe a new high specificity and high sensitivity HT screening assay. It is based on the expression of a gene induced by chromosome mutation, breakage and mis-segregation in human cells (GADD45a). The GFP based reporter system is amenable to microplate assay as well as flow and possibly scanning cytometric assay formats. It has effective coverage of all the major mechanisms of genotoxicity, accompanied by high levels of specificity and sensitivity in the detection of genotoxic carcinogens (Hastwell *et al.*, 2006).

Background.

Kirkland *et al* (2005) have drawn attention to the poor specificity of the regulatory *in vitro* mammalian genotoxicity tests. This leads to many confounding false indications of potential cancer hazard – and for an early screening program, the loss of potentially valuable compounds. The graph below (made by Andrew Scott, Unilever, and drawn from that paper) clearly reveals the apparent trade off between sensitivity and specificity in the individual tests and their combinations.



There are simple explanations for the low sensitivity of the Ames test: the bacterial cells lack many of the eukaryotic targets for genotoxins, including chromatin DNA, the enzymes involved in replication and repair that interact with chromatin, and the mitotic apparatus.

It is less clear why the mammalian cell tests are over-sensitive. Kirkland and coworkers (2006) have ruled out simple reasons such as toxicity, magnitude of response and lowest effective positive concentration.

It is possible that the properties of some of the commonly used cell lines might contribute to the problem. These cell lines (by selection) have defects which allow them to divide in an unregulated way. The development of cancers itself reflects similar defects, such as failure to correctly trigger apoptosis, induce DNA repair or exit the cell cycle.

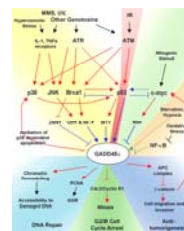
The GADD45a gene is a transcriptional target of many key tumour suppressors and oncogenes.

Red connectors indicate an effect on protein activity.

Blue connectors indicate an effect on transcriptional activity.

Green connectors indicate an effect on mRNA stability.

...Dotted connectors indicate that the mechanism of regulation is unclear.



p53 dependent GADD45a expression

The promoter of the GADD45a gene has complex regulation, but key to the development of the reporter was our discovery that a putative p53 element in intron 3 is critical to the correct response. For this reason we have chosen the p53 competent, human lymphoblastoid cell line TK6 as an initial host.

Resolving data conflicts

- Hastwell *et al.*, 2006: This study included 9 toxicity associated, 'unique' mammalian *in vitro* positives. All were negative in GreenScreen HC and *in vivo* studies.

- Manuscript in preparation: 62 further marketed pharmaceuticals. Taking data from both studies we have tested 43 Ames negative, *in vitro* mammalian cell positive compounds.

28 of 43 tested negative in the *in vivo* MNT and 26 of these were correctly negative with GreenScreen HC. The 2/28 HC positives are Cyclopirox Olamine, a possible *in vitro* clastogen and Griseofulvin an aneugen, where the low *in vivo* exposure data might account for the negative result.

15 were positive in the *in vivo* MNT, and 11 were correctly positive in GreenScreen HC. The 4/15 negatives are Thiabendazole: *in vivo* unreplicable; 2,4-Dichlorophenol: high level of robertsonian translocations; Pyrazinamide: weak response *in vivo*; Stavudine: nucleoside analogue, only *in vivo* positive at high concs.

All genotoxic mechanisms detected

10/10	direct acting agents	NQO, MMS, Cisplatin, MNNG...
4/4	topo II inhibitors	Etoposide, Camptothecin...
9/10 ¹	aneugens	Benomyl, Colchicine, Paclitaxel...
7/8 ²	nucleotide synthesis inhibitors	AZT, 5FU, Aphidicolin...
2/3 ³	ROS, positive	Bleomycin, hydrogen peroxide
3/3 ⁴	S9/promutagens	BaP, DMBA, cyclophosphamide

¹ not thiabendazole, see above;
³ not paraquat, phase II deficiency?;

² not didanosine, equivocal in battery
⁴ proof of principle, alternate protocol

Predictivity measures.

(Data from 75 compounds, Hastwell *et al.*, 2006)

1. Carcinogenicity

assay	all carcinogens	genotoxic carcinogens
Ames	82%	92%
MLA	77%	88%
In vitro CA	69%	79%
In vivo CA	81%	89%
GADD45a	86%	98%

2. ICH battery tests:

(All results negative = negative; any positive = positive)

Excluding <i>in vitro</i> 'false' positives	Concordance	94%
	Sensitivity	88%
	Specificity	100%

3. Individual tests, all data (including 'false' positives)

	Sensitivity	Specificity
Ames	88%	75% gets Ames +ves correct
MLA	81%	100% gets the all-too-rare
<i>in vitro</i> CA	72%	100% <i>in vitro</i> -ves correct
<i>in vivo</i> MNT	73%	84%

Summary

The new GADD45 assay effectively detects gene and genome damage

The assay is at least effective as any of the current regulatory tests, and has much higher sensitivity in the validation set of 75 compounds

The 96 well microplate format, low compound requirement and 48 hour endpoint make the assay suitable for EARLY SCREENING

The high specificity of the assay provides a potentially powerful tool in the resolution of data conflicts between regulatory genotoxicity tests

References

Hastwell, L. Chai, K. Roberts, T. Webster, J. Harvey, R. Rees, and R. Walmsley. High specificity and high sensitivity genotoxicity assessment in a human cell line: validation of the GADD45a-GFP genotoxicity assay. *Mutation Research*, 607 (2006) 160-175.

D. Kirkland, M. Aardema, L. Henderson and L. Muller. Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens: I. Sensitivity, specificity and relative predictivity. *Mutation Research*, 584 (2005) 1-256.

D. Kirkland, M. Aardema, L. Henderson and L. Muller. Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens: II. Further analysis of mammalian cell results, relative predictivity and tumour profiles. *Mutation Research* 608 (2006) 29-42