

How Does Increasing Cytotoxicity Affect The Accuracy Of The *GADD45a*-GFP (GreenScreen HC) Genotoxicity Screening Assay: A Comparison of Different Methods To Estimate Cytotoxicity

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Introduction

Recent evaluations of the *in vitro* mammalian genotoxicity assays have suggested that increased levels of cytotoxicity may increase the prevalence of uniquely positive genotoxicity results [1,2]. Retrospective studies have shown these assays to be misleading in their prediction of *in vivo* genotoxicity and rodent carcinogenicity results at high levels of cytotoxicity [1,2,3]. These findings have rightly focused attention on the different methods used to assess cytotoxicity in the *in vitro* mammalian genotoxicity assays [4,5,6].

The *GADD45a*-GFP *in vitro* genotoxicity assay is a 96-well microplate-based screening assay that has been shown to have sensitivity comparable to other *in vitro* mammalian tests but coupled with much higher specificity [6]. During the validation of the assay, relative cell density (RCD) was used as a measure of cytotoxicity and a limit for data inclusion was set at 30% RCD. The experiments presented here have compared other measures of cytotoxicity in cells exposed to toxins that have been shown to reduce RCD. These included measures currently proposed for accurately estimating cytotoxicity in the *in vitro* micronucleus test [5,6]. Furthermore, it is considered whether RCD, calculated using optical density, provides an accurate assessment of cytotoxicity in the *GADD45a*-GFP assay.

Methods 1

The *GADD45a*-GFP assay was used to assess 16 compounds over 9 serial dilutions for genotoxic hazard after 48hrs incubation. The 16 compounds tested included 8 genotoxic (see Table 1) and 8 non-genotoxic compounds (see Table 2) which were tested in triplicate in the *GADD45a*-GFP assay. At the 48hr time point the following 4 methods for estimating cytotoxicity were conducted: RCD, propidium iodide exclusion (PI), intracellular ATP levels (ATP) and cell number estimation (CE, using a fluorescent DNA stain). Data from these experiments are shown in Results 1 below.

Results 1

- The levels of cytotoxicity estimated using RCD, ATP, PI and CE all differed for all compounds inducing cytotoxicity (see Tables 1 & 2 and Figure 1).
- All 8 genotoxins had percentage survivals of >30% when measured by RCD, although 2 compounds reduced survival to <30% when measured by PI or ATP, (see Table 1).

Compound	Test Concentration (µg/ml)	Genotoxicity LEC (µg/ml)	Percentage Survival at the Highest Test Concentration			
			Estimated by RCD	Estimated by PI	Estimated by ATP	Estimated by CE
Methylnitrosourea	1030	64.38	75.69	7.19	14.80	No Test
4-Nitroquinoline-N-oxide	1	0.25	75.43	34.06	46.46	57.50
Camptothecin	0.1	0.003	84.20	32.01	65.48	73.60
Etoposide	8	0.50	82.98	66.29	102.32	58.80
Paclitaxel	0.5	0.06	86.41	57.18	78.79	71.20
Vincristine sulphate	0.1	0.0004	68.53	29.10	60.33	58.80
5-Fluorouracil	20	2.50	75.16	73.73	90.10	56.20
Aphidicolin	8.5	0.27	83.24	40.35	76.05	57.70

Table 1. Cytotoxic effects at the genotoxicity LEC – assessed using RCD, ATP, PI and CE. Mean data (n=3) presented for 8 known genotoxic compounds at the 48hr endpoint of the *GADD45a*-GFP assay.

- Four of the non-genotoxic compounds had little or no inhibitory effects on cell survival and a high level of agreement was seen between the 4 methods for these compounds.
- For the 4 cytotoxic non-genotoxins, RCD was shown to underestimate the levels of cytotoxicity occurring: 3 compounds that recorded percentage survival of >30% by RCD were found to demonstrate survival levels of <1% when measured using PI and ATP, (see Table 2).

Compound	Test Concentration (µg/m)	Genotoxicity LEC (µg/ml)	Percentage Survival at the Highest Test Concentration			
			Estimated by RCD	Estimated by PI	Estimated by ATP	Estimated by CE
Chloramphenicol	1616	-	56.90	0.12	0.00	40.10
2,4-Dichlorophenol	324	-	35.90	0.15	0.00	No Test
2,4-Dinitrophenol	1840	-	45.02	0.14	0.55	31.70
Phenformin HCl	242	-	59.58	27.82	21.94	41.40
Ampicillin	3710	-	103.95	101.85	113.24	107.50
Ethylene glycol	621	-	102.65	99.87	101.67	102.20
D-Mannitol	1822	-	99.27	98.89	102.99	102.30
Sodium chloride	585	-	101.85	99.59	105.31	102.20

Table 2. Cytotoxic effects at the highest concentration tested – assessed using RCD, ATP, PI and CE. Mean data presented (n=3) for 8 known non-genotoxic compounds at the 48hr endpoint of the *GADD45a*-GFP assay.

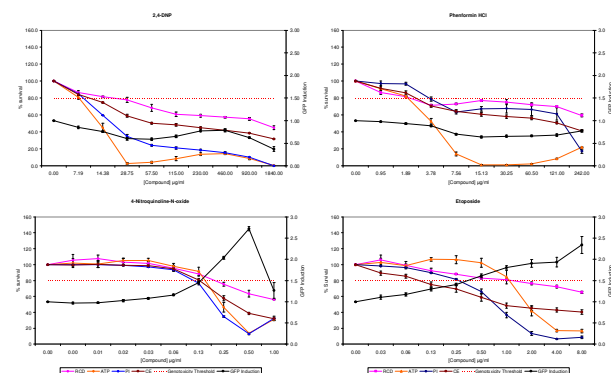


Figure 1. Representative data demonstrating the comparison of 4 cytotoxicity methods at the 48hr endpoint of the *GADD45a*-GFP assay. Genotoxicity dose-response curves from the *GADD45a*-GFP assay are also shown for each compound. Data points are the mean value from n=3, with error bars showing 1 S.E.M.

Conclusions

- Data from this investigation show that the *GADD45a*-GFP assay is **compatible with alternative cytotoxicity methods** and that additional cytotoxicity data can be readily collected following the 48 hour assay endpoint.
- Relative cell density appears to underestimate cytotoxicity when compared with other measures. Indeed, some cytotoxic, non-genotoxic chemicals that generated an RCD above the 30% genotoxicity data inclusion limit exhibited <10% percentage survival when measured by PI exclusion or intracellular ATP levels and 0% survival when assessed by RPD.
- Importantly, these compounds generated **negative results for genotoxicity even when tested to very high levels of cytotoxicity**. The work presented here suggests that cytotoxicity alone does not activate the *GADD45a*-GFP reporter.

Methods 2

For a subset of the 16 compounds tested, 4 compounds were also tested in the *GADD45a*-GFP assay over 4 concentrations and cytotoxicity was assessed using the following 3 methods with results compared to those for RCD (see Results 2 below):

Relative population doublings (RPD)

$$\text{Population Doubling (PD)} = \text{Log}(48\text{hr cell count}^* / \text{starting cell count}) / \text{Log}(2)$$

$$\text{RPD} = (\text{PD treated sample} / \text{PD untreated control}) \times 100$$

Relative increase in cell counts (RICC)

$$\text{RICC} = \frac{(48\text{hr cell counts}^* \text{ treated sample} - \text{starting cell density})}{(48\text{hr cell counts}^* \text{ untreated sample} - \text{starting cell density})} \times 100$$

Relative suspension growth (RSG)

$$\text{Suspension growth (SG)} = \frac{24\text{hr cell count}^*}{\text{Starting density}} \times \frac{48\text{hr cell count}^*}{24\text{hr cell count}^*}$$

$$\text{RSG} = (\text{SG for treated sample} / \text{SG of untreated control}) \times 100$$

*cell counts by haemocytometer and only viable cells included (assessed by trypan blue exclusion)

Results 2

- For compounds which induced cytostasis/cytotoxicity in the *GADD45a*-GFP cells, RCD consistently underestimated the levels of toxicity compared to RPD, RICC and RSG (see Figure 2).
- For Taxol, a well characterised genotoxic compound, the level of survival at the LEC for genotoxicity when measured by RPD and RICC was 0% compared to 78% when measured by RCD. A similar result was also seen for NQO where the level of survival at the LEC for genotoxicity was <30% when measured by RPD and RICC compared to >70% when measured by RCD.
- For Phenformin HCl, a non-genotoxic compound, a similar underestimation of cytotoxicity is seen by RCD however there is no induction of the *GADD45a*-GFP reporter.

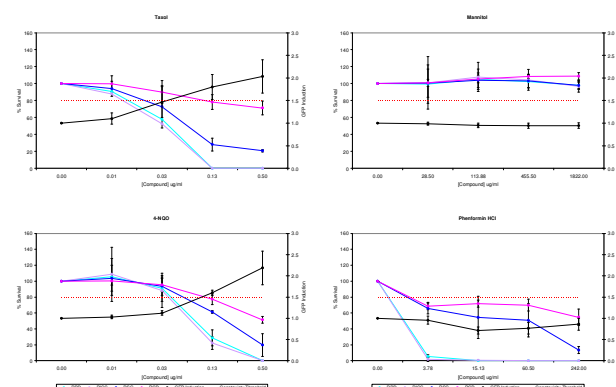


Figure 2. Representative data demonstrating the comparison of RCD with 3 cytotoxicity methods routinely used in the regulatory *in vitro* mammalian genotoxicity assays at the 48hr time point of the *GADD45a*-GFP assay. Genotoxicity dose-response curves from the *GADD45a*-GFP assay are also shown for each compound. Data points are the mean value from n=3, with error bars showing 1 S.D.