

**COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT (COM)**

**GADD45a-GFP 'GreenScreen HC' Genotoxicity Assay: An Update**

**1) Introduction:** The Committee was introduced to the TK6 GADD 45a assay during 2007 when Professor Walmsley gave a comprehensive talk on this newly developed high-throughput *in vitro* genotoxicity assay. The data were reported by Hastwell et al (2006). These early screens gave very promising results and the Committee advocated the development of new assays such as this which, have good sensitivity (correct identification of positives) and specificity (correct identification of negatives).

2) Briefly, the assay utilises *GADD45a*, a gene thought to play a role in DNA repair, cell cycle control and apoptosis in response to genotoxic insult. Induction of *GADD45a* has been identified in early gene expression microarray experiments in response to a wide range of genotoxins (e.g direct DNA damaging, topoisomerase inhibitors, nucleotide synthesis inhibitor, aneugens, reactive oxygen generators) in various cell types. This increase in *GADD45a* suggested that it could be utilised as a suitable marker gene for genotoxic stress. In this system *GADD45a* is fused to a Green Fluorescent Protein (GFP) gene, the plasmid construct is transfected into a p53 proficient human lymphoblastoid cell line (TK6) and the assay is conducted in microplates. Cells (exponential phase cultures) are incubated for 24 h and 48 h with serial dilutions of test compounds, after which GFP reporter fluorescence and cell culture absorbance are measured. A number of decision thresholds are defined; acceptance of the assay is based upon a statistically significant reduction in proliferation potential or relative suspension growth set at 80% maximum growth, which is greater than 3 times the standard deviation of the background brightness. A threshold for cytotoxicity is set at 30%, and for genotoxicity a statistical significant increase in brightness greater than threshold of GFP of 1.5 is considered positive. In this initial study 75 chemicals were selected for testing, 34 were genotoxic by a variety of mechanisms and the utility of this assay for genotoxicity testing was demonstrated.

3) Since this original presentation, a number of significant studies have been conducted which have aimed to further validate the assay and to introduce modifications to the assay, most significantly a protocol using metabolic activation and a higher throughput schedule. An overview detailing these developments has been drafted by Professor Walmsley and is included as Annex 1. Several articles discussed are in press. These, together with other recently published papers, are included in Annex 2 for your attention.

**4) Inter-laboratory validation:** An important aspect of assay validation is described by Billington et al (2008) who undertook an inter-laboratory comparison assessment of the GADD45a GFP 'GreenScreen' assay. Sixteen

chemicals were tested in 4 different laboratories, each chemical tested on 4 different occasions. It was shown that 92% of the assays gave the expected results. It is noteworthy that only chemicals which did not require metabolic activation were used. The study demonstrated that the assay transferred effectively to new laboratories and that the overall concordance was 92.5%. The ranges for sensitivity and specificity were 86.4-100% and 66.7-94.1% respectively.

**5) Metabolic activation:** The development of the assay to incorporate a metabolic activation step is detailed by Jagger et al (2009). In the original assay system the spectrophotometric fluorescence detection system is ineffective in the presence of S9 which interferes with the fluorescence measurements. Modifications to the method were made as follows: the addition of S9 mix at a final concentration of 1% S9; incubated for 3 hours; cells harvested by centrifugation of the microplates, 2x washes resuspended in PBS containing propidium iodide (PI). Flow cytometry (FCM) was used to measure intracellular GFP and the inclusion of PI enables cellular viability measurements. An induction of 1.3-fold GFP was considered positive, based on the 3 times the SD as established for the assay without S9. An 80% toxicity limit was set for the rejection of data. It is predicted that FCM will also minimize interference from light-absorbing compounds.

6) A total of 56 chemicals were used to validate the screen, 25 of which were expected to be pro-genotoxins and also 'known' or 'reasonably anticipated' to be human carcinogens according to NTP Report on Carcinogens (see Table 1 in the paper). Each chemical was tested 3 times. Of the 25 pro-genotoxins, 20 generated positive results. 2,4-DAT, disperse orange, hexamethylphosphoramide, isoprene and MeIQ gave the unexpected negative results. The authors concluded that the method permitted detection of the majority of pro-genotoxins with a high specificity. However, Members are asked to consider in detail of the FCM methodology with regards to its robustness and sensitivity.

**7) Further general validation:** In a recent, comprehensive study, 75 commercially available pharmaceutical compounds, chosen to cover a range of therapeutic indications were tested (Hastwell et al, in press). The assay incorporating S9 was not used in this study. Compounds covering a wide variety of therapeutic indications were evaluated, including 11 compounds which were considered to have given false positives, and the results were compared with the existing genotoxicity and carcinogenicity data-set. The results showed a concordance of 87%, 93% and 66% with *in vivo* genotoxicity data, genotoxic carcinogenicity and carcinogenicity

8) An independent evaluation of GADD45a- GFP assay has been presented by Olaharski et al (2009). Ninety one compounds were tested, 57 classified as *in vitro* genotoxins and 34 non-genotoxic. Of these, 50 were the non-proprietary set of chemicals (33 were rodent carcinogens) and 41 were Roche proprietary compounds which had been assessed in the Ames test and mouse micronucleus assays, 26 of which had been declared to be genotoxic. Rodent carcinogenicity data was available for only one of these compounds. The compounds were tested using the protocol described in Hastwell et al

(2006), either at Bioreliance or at Genotronix. No compounds which required metabolic activation were included in this analysis. The thresholds of 80% reduction in proliferation potential and 1.5-fold induction of GADD45a were used. *In vitro* micronucleus assays in mouse lymphoma cells and a microplate Ames test were also conducted on the elected compounds and the concordance with different end-points was addressed. Of the genotoxic compounds 20/26 proprietary compounds and 19/31 non-proprietary compounds were declared to have been accurately predicted by the GADD45a GFP assay. From these analyses, sensitivity and specificity for genotoxicity was shown to be 30% and 97% respectively and for carcinogenicity, 30% and 88% respectively. The authors conclude that the assay is robust and reproducible, with low sensitivity and high specificity.

9) Recently the results from a substantial trial evaluating the ECVAM recommended lists of chemicals became available (Birrell et in press), the project aims to reduce the number of false positives. A total of 62 chemicals were tested; these had been rigorously selected to fall into three categories. Eight were expected to require metabolic activation. The protocols with and without S9 were used.

10) The following results were obtained;

- Group 1 : chemicals that should be detected as positive in *in vitro* mammalian cell genotoxicity tests ; 18/20 (90%) were reproducibly positive
- Group 2: chemicals that should be detected as negative in *in vitro* mammalian cell genotoxicity tests ; 22/23 (96%) were reproducibly negative.
- Group 3; chemical which should give negative results in mammalian *in vitro* genotoxicity tests but have been reported to induce chromosomal aberrations or TK mutations at high concentration/cytotoxicity. 13/17 (76%) were reproducibly negative

Again high specificity was demonstrated for the GADD45a-GFP assay.

11) **Higher throughput protocol:** The assay has been developed to use a robotic liquid handling automation system, where 12 compounds can be tested per 96 microwell plate (Knight et al 2009). In this study 1266 diverse, pharmacologically active compounds were tested, and the results generated were compared to DEREK analyses. 46 of the 92 compounds that were positive in the GreenScreen produced genotoxicity alerts in DEREK. However of 50 chemicals which had positive carcinogenesis data, GreenScreen alerted for only 20% of these compared to DEREK which alerted for 56%.

12) Recently this high-throughput version of the GADD assay was utilised as part of the US EPA ToxCast programme. (Knight et al, in press). This programme aims to use data from *in vitro* high throughput screening (HTS) assays to contribute to risk assessment strategies by providing insight into mechanisms of action of potential toxicants. A number of different HTS were evaluated including the GreenScreen. Three hundred and twenty were

tested, sensitivity and specificity as compared to identification of Ames positives and *in vivo* tumourigenic endpoints were reported. Sensitivity was low (11.6-15.2%) whereas specificity was high 90-94.4%

### **Summary:**

13) Since the first presentation given by Professor Walmsley to COM on the GADD 45a GreenScreen assay a significant body of data has been generated which contributes towards its validation. The assay appears to perform robustly, as shown by the inter-laboratory investigation. The method was adapted to use S9 and currently this arm of the assay would appear to be less well validated. The very high-throughput development has been used successfully in the ToxCast Programme.

14) Generally the assay has been shown to have high specificity and sensitivity and appears to reduce the number of false positives, a big aim during the development of new assays. However, it is noteworthy that there was some controversy surrounding the results of the Olaharski study which suggested a lower sensitivity. This is discussed in Walmsley and Billington (2009) and members are asked for their opinion on this commentary.

### **Questions for Committee Members;**

- What are members' general views on the GADD45a assay?
- What are members' views of the data submitted for the validation of the GADD45a assay including the choice of chemicals for testing?
- What are members' views on the assay using exogenous metabolic activation?
- What are members' views on the submitted information on inter-laboratory validation and high-throughput screening?
- What further data are required to validate the assay, and what future purpose could it serve in genotoxicity testing?
- Where would members place this assay in the COM strategy for testing mutagenicity?

DH Toxicology Unit: September 2009

### **References;**

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