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GADD45a-GFP GreenScreen HC assay results for the ECVAM recommended lists of genotoxic and non-genotoxic chemicals for assessment of new genotoxicity tests

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ABSTRACT

A recent ECVAM workshop considered how to reduce falsely predictive positive results when undertaking *in vitro* genotoxicity testing, and thus to avoid unnecessary follow-up with tests involving animals. As it was anticipated that modified versions of existing assays as well as new assays might contribute to a solution, an expert panel was asked to identify a list of chemicals that could be used in the evaluation of such assays. Three categories of test chemicals were chosen comprising a total of 62 compounds. This paper provides test results for these chemicals using the GreenScreen HC assay. All tests were carried out in triplicate, by multiple operators, with and without S9, using invariant protocols. Group 1 chemicals should be detected as positive in *in vitro* mammalian cell genotoxicity tests: 18/20 (90%) were reproducibly positive in GreenScreen HC. Group 2 chemicals should give negative results in *in vitro* genotoxicity tests: 22/23 (96%) were reproducibly negative in GreenScreen HC. Overall concordance for Groups 1 and 2 is 93%. Group 3 chemicals should give negative results in *in vitro* mammalian cell genotoxicity tests, but have been reported to induce chromosomal aberrations or *Tk* mutations in mouse lymphoma cells, often at high concentrations or at high levels of cytotoxicity: 13/17 (76%) were reproducibly negative in GreenScreen HC. Of the four positive compounds in Group 3, *p*-nitrophenol was only positive at the top dose (10 mM), 2,4-DCP is an *in vivo* genotoxin, and two chemicals are antioxidant compounds that may be acting as pro-oxidants in the hyperoxic conditions of cell culture. Overall, these predictive figures are similar to those from other studies with the GreenScreen HC assay and confirm its high specificity, which in turn minimizes the generation of falsely predictive positive results.

1. Introduction

It has been recognized in at least three large studies that the regulatory *in vitro* mammalian genotoxicity assays can generate positive genotoxicity data that are neither predictive of an *in vivo* genotoxic hazard nor of a carcinogenic hazard, i.e. they produce positive results for compounds that are neither *in vivo* genotoxins nor carcinogens - so-called 'false' or 'misleading' positives [1–3]. It follows from this that novel compounds, which might be candidate pharmaceuticals, industrial chemicals, cosmetics or household products, can produce positive results in these tests but not pose a genotoxic or carcinogenic hazard. Since such compounds can contribute to the welfare of animals, improve human health, and be economically profitable, 'follow up' testing in animals is carried out as part of an overall safety/risk assessment. Ironically then, animal tests are used to decide whether or not the *in vitro* results are reliable. Manufacturing sufficient material for these animal tests is costly, and – perhaps equally important – the dosing of animals with compounds that are likely to be hazardous is unacceptable to many. It has also been recognized that the *in vivo* tests can produce a false prediction of carcinogenicity, albeit more rarely [4]. An ECVAM workshop [5] addressed the critical need of both industry and the public to reduce false positives in the *in vitro* assays and avoid unnecessary animal tests. The workshop made recommendations for improvements or modifications to existing tests, and considered new skin models and cell systems, including the then new GreenScreen HC assay. The workshop convened an expert panel to make recommendations for chemicals that could be used in the evaluation of modified or new assays [6]. Their deliberations produced three lists, comprising a total of 62 compounds: Group 1 chemicals should be detected as positive in *in vitro* genotoxicity tests in mammalian cells. Group 2 chemicals should give negative results in *in vitro* mammalian genotoxicity tests. The similar numbers of carcinogens/genotoxins and non-carcinogens/non-genotoxins in

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Groups 1 and 2, respectively, provide a good balance for concordance calculations. Group 3 chemicals should give negative results in *in vitro* mammalian genotoxicity tests, but have been reported to induce chromosomal aberrations or *Tk* mutations in mouse lymphoma cells, often at high concentrations or at high levels of cytotoxicity.

The GreenScreen HC genotoxicity assay monitors genotoxin-induced transcription of the *GADD45a* gene using an in-frame reporter gene encoding the Green Fluorescent Protein (GFP) [7]. *GADD45a*, originally identified and named by the Fornace laboratory [8], has been implicated in the response to genome damage by genetic, biochemical and genomic approaches [8–13]. Mice lacking the gene are more prone to tumours induced by ionizing radiation and genotoxins [9]; their lymphoblasts and fibroblasts have defective nucleotide excision repair; their fibroblasts show centrosome amplification and unequal segregation of chromosomes due to multiple spindle poles and the induction of aneuploidy [10]. The Gadd45a protein modifies DNA accessibility in damaged chromatin and associates with nuclear factors involved in cell-cycle regulation [11,12]. In microarray studies, the gene is one of those most robustly induced by genotoxins [13]. *GADD45a* was the first gene to be identified as a target of p53 [14], and has key roles in cell-cycle regulation and DNA repair [15] as well as apoptosis [16]. These studies all implicate *GADD45a* as a clear component of the pathways that contribute to the maintenance of genomic stability following genotoxic stress, and this is reflected in its induction by mutagens, clastogens and aneugens [7,17].

The *GADD45a* reporter is hosted by the human lymphoblastoid cell line TK6, which is p53-competent – a necessary attribute for a proper genotoxic response in all mammals. For detailed information readers are referred to the original validation paper [7], the subsequent transferability 'ring trial' [18], the validation of a protocol for the assessment of S9 metabolites, which completed the menu of genotoxins identified by the assay [19], and to some larger studies including the 1266 compounds from the "Sigma Library of Pharmacologically Active Compounds" [20], 320 compounds comprising the ToxCast EPA Phase 1 collection [21] and 75 marketed pharmaceuticals [22]. Additional data have been generated from the >8000 compounds from the 60 proprietary collections of pharmaceutical and biotechnology companies and laboratories that have used the assay in the early identification of genotoxic hazard.

A data collection as large as this one generates fairly robust summary data for the analysis of potential genotoxic hazard in compound libraries. In the largely "unselected" collection of chemicals in the compound libraries listed above, positive prevalences of GreenScreen HC data are partly a function of the highest concentration tested. This concentration is largely determined by the need to dilute library samples, which are generally held in 95–100% DMSO, to the 1% (v/v) DMSO tolerated by the reporter cells. In the LOPAC collection [20] screening at 100 μM generated about 7% positives; in the ToxCast collection screening at 200 μM generated about 10% positives [21]. This is a little lower than the 12% of drug candidates that raise genotoxicity safety concerns following the battery of genotoxicity tests [23], carried out at concentrations up to 10 mM.

The initial validation studies focused on those carcinogens/genotoxins that cause the major classes of genotoxic damage, and in contrast to the larger studies mentioned above (LOPAC and ToxCast), compounds were available at sufficient concentrations to allow testing up to current ICH S2B dose-requirements: 10 mM or 5 mg/ml, or lower if solubility or toxicity limited the testing. In these validation studies the GreenScreen HC assay demonstrated a high sensitivity to mutagens, clastogens and aneugens of all mechanistic classes. The studies included approximately equal numbers of non-carcinogens/non-genotoxins, and the overwhelming majority of these were negative. For the 109 compounds where the observed carcinogenicity is commonly agreed to be due to a genotoxic mode of action, the sensitivity of this assay to genotoxic carcinogens is 87% and the specificity is 95%. This high specificity is a key attribute required of any new genotoxicity test, and reflects a low incidence of false-positive prediction of cancer hazard. These figures include data from a study by Olaharski et al. [24,25].

Performance of an *in vitro* assay can also be judged by its prediction of *in vivo* genotoxicity data. Data on chromosomal aberrations *in vivo* have now been collected for 133 compounds tested with the GreenScreen HC assay. The sensitivity and specificity of the assay to *in vivo* genotoxins are 78% (40/49) and 94% (80/84), respectively.

Despite this large volume of test results, developers of any new assay are constantly asked for more data to support their widespread use. For genetic toxicologists the generation of a definitive list of compounds to assess performance has seemed an almost impossible task, so for those seeking to reduce irrelevant positive results by the introduction of new assays, the 'recommended' list [6] provides a very useful and very welcome reference point.

The principal aim of this work was to provide GreenScreen HC data for the recommended list of compounds [6] to allow its comparison with other tests. A secondary aim was to discover whether the performance of the assay in terms of sensitivity and specificity in this collection was consistent with the performance figures derived from other studies that used this assay.

2. Materials and methods

2.1. Selection of compounds

Compounds were sourced at the highest purity available in the UK. A number of these compounds had already been tested in either the original GreenScreen HC validation study without S9 (denoted by '*' in Tables 1–3) [7] or in the validation study of the S9 method (denoted by '§' in Tables 1–3) [19] and these data have been included in this paper. The majority of compound data are new. Ephedrine was tested in the first validation study for GreenScreen HC [7] where it gave a negative result without S9. However, it is now subject to purchasing restrictions in the UK, and could not be obtained by our laboratory for S9 testing. Ethyl acrylate was not tested due to its high volatility.

2.2. The assay method

The assay protocols have been described fully elsewhere, so they are summarized here. All compounds were tested in a standardized 96-well format [7], in which four compounds are each tested in two series of nine two-fold dilutions. One series is tested using the TK6 cell line expressing the GADD45a-GFP reporter (test cells), and the second is tested using a TK6 cell in which the reporter has a non-expressed GFP gene (control cells). Data from the control cells identify compounds that are themselves fluorescent, or induce cellular autofluorescence, and also allow for the subtraction of such confounding data from the test-cell data. Data from S9-treated samples were collected by use of flow cytometry [19], and data from samples not treated with S9 were collected either using fluorescence/absorbance spectrometry [7] or flow cytometry. The two methods give the same results for compounds not requiring S9 [19].

Compounds were tested in a final DMSO concentration of 1% (v/v water) with the exception of sodium arsenite, which was tested in water alone because DMSO is a confounding chemical factor for the genotoxicity of this compound [26]. All compounds have been tested at least three times, both with and without S9. The compounds were not coded and were prepared and tested by a panel of four operators. Three replicates for each compound were tested on different microplates. Each compound was freshly prepared for each replicate assay. All compounds were tested using a final concentration 1% S9 derived from Aroclor 1254-treated rats (Moltox).

Positive controls for tests without S9 were two different concentrations of MMS; positive controls for tests with S9 were two different concentrations of cyclophosphamide. For data acceptance, the two doses must show appropriate induction. In addition there should be no contamination evident in cell-free medium controls. GFP fluorescence is normalized to cell numbers to give a brightness figure, and a positive result is noted where this is greater than or equal to a threshold value of 1.5 times the value of the vehicle-treated control for the spectrophotometric method, or 1.3 times the vehicle-treated control for the flow cytometry method. These thresholds are greater than three times the S.D. in the mean GFP signal from a large number of assays with non-genotoxic toxins and non-toxins. The thresholds are different for the two assay types because in the microplate method, measurements of the fluorescence and absorbance data include the contribution of cells, medium, test compound and the microplate itself. In contrast, in the case of flow cytometry the