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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

Louise Birrell^a, Paul Cahill^a, Chris Hughes^a, Matthew Tate^{a,b}, Richard M. Walmsley^{a,b,*}

^a Gentronix Ltd, 46 Grafton Street, Manchester M13 9NT, UK

^b Faculty of Life Sciences, The University of Manchester, Manchester M13 9PT, UK

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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.

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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 Groups 1 and 2, respectively, provide a good balance for concor-

* Corresponding author at: Faculty of Life Sciences, The University of Manchester, Manchester M13 9PT, UK. Tel.: +44 161 6037662; fax: +44 161 6067337.

E-mail addresses: louise.birrell@gentronix.co.uk (L. Birrell), paul.cahill@gentronix.co.uk (P. Cahill), chris.hughes@gentronix.co.uk (C. Hughes), matthew.tate@gentronix.co.uk (M. Tate), richard.walmsley@manchester.ac.uk (R.M. Walmsley).

dance 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 contributes 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 prevalence 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 μ M generated about 7% positives; in the ToxCast collection screening at 200 μ M 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 insufficient 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 micro-plates. 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 data are collected on viable cells only, and interference from medium/test com-

Table 1*In vivo* genotoxins that should be detected as positive in *in vitro* mammalian cell genotoxicity tests.

Chemical	Test results without S9			GADD45a-GFP genotoxicity	L.E.C. (μg/ml)	L.E.C. (μM)	Test results with S9		Test con- centration limitation	GADD45a- GFP genotoxicity	L.E.C. (μg/ml)	L.E.C μM	Lowest L.E.C. (μM)	L.E.C. rank
	Top test conc. (μg/ml)	Top test conc. (mM)	Test concentration limitation				Top Test Conc. (μg/ml)	Top Test Conc. (mM)						
(I) Ames-positive <i>in vivo</i> genotoxins														
(i) O ⁶ and N7 alkylators														
25.00 Cyclophosphamide [§]	0.09	Cyto	N	–	25.00	0.09	Cyto	P	6.25	22.39	22.39	10		
1200.00 ENU (N-Nitroso-N-ethyl urea) [§]	10.25	mM	P	125.00	1067.37	1171.10	10.00	mM	P	36.60	312.50	312.50	17	
50.00 MMS [§]	0.45	Cyto	P	25.00	227.00	50.00	0.45	Cyto	P	6.25	56.25	56.25	11	
(ii) Polycyclic aromatic hydrocarbons														
20.00 Benzo[a]pyrene [§]	0.08	Sol	N	–	20.00	0.08	Sol	P	1.25	4.96	4.96	7		
20.00 7,12-Dimethylbenzanthracene [§]	0.08	Sol	P	2.50	10.00	20.00	0.08	Sol	P	1.25	4.88	4.88	6	
(iii) Aromatic amines														
741.00 Dimethylnitrosamine [§]	10.00	mM	N	–	741.00	10.00	mM	P	46.31	624.97	624.97	19		
200.00 2-Acetylaminofluorene [§]	0.90	Sol	N	–	200.00	0.90	Sol	P	50.00	223.94	223.94	15		
250.00 2,4-Diaminotoluene [§]	2.00	Sol	P	31.25	255.75	250.00	2.00	Sol	N	–	255.75	16		
240.00 IQ [§]	1.21	Sol	P	60.00	302.66	240.00	1.21	Sol	P	30.00	151.34	151.34	13	
160.00 PhIP [§]	0.71	Sol	P	80.00	356.73	160.00	0.71	Sol	P	1.25	5.57	5.57	8	
(iv) Others														
10.00 Aflatoxin B1 [§]	0.03	Cyto	P	10.00	32.02	10.00	0.03	Cyto	P	0.04	0.12	0.12	4	
9.10 Cadmium chloride	0.04	Cyto	N	–	14.32	0.08	Cyto	P	1.79	9.76	9.76	9		
16.00 Cisplatin [§]	0.05	Cyto	P	1.00	3.33	375.10	1.25	Cyto	P	5.86	19.53	2.44	5	
1276.00 <i>p</i> -Chloroaniline	10.00	mM	N	–	1276.00	10.00	mM	N	–	–	–	–	–	
(II) <i>In vivo</i> genotoxins negative or equivocal in Ames														
8.00 Etoposide [§] , [§]	0.01	Cyto	P	0.06	0.10	8.00	0.01	Cyto	N	–	–	0.06	3	
12.30 Hydroquinone	0.11	AF	N	–	1101.00	10.00	mM	P	8.60	78.11	78.11	12		
2670.00 Azidothymidine [§]	9.99	mM	P	1335.00	4995.00	2672.40	10.00	mM	N	–	–	5000.00	21	
20.00 Sodium arsenite	0.00	Cyto	P	0.63	0.01	129.91	0.00	Cyto	N	–	–	0.01	1	
4.00 Taxol [†] , [§]	0.01	Cyto	P	0.02	0.03	0.50	0.00	Cyto	P	0.06	0.08	0.03	2	
3231.00 Chloramphenicol [†]	10.00	mM	N	–	3231.00	10.00	mM	N	–	–	–	–	–	

[†] Data from testing without S9 extracted from [7].[§] Data from testing with S9 extracted from [19] (except 2,4-diaminotoluene for which data for $\pm$ S9 testing were taken from [19]).

Table 2

Non-DNA reactive chemicals (including non-genotoxic carcinogens) that should give negative results in *in vitro* mammalian cell genotoxicity tests.

Chemical	Test results without S9			GADD45a-GFP genotoxicity	Test results with S9		Top test conc. (µg/ml)	Top test conc. (mM)	Test con- centration limitation	GADD45a- GFP genotoxic- ity	L.E.C. (µg/ml)	L.E.C (µM)	Lowest L.E.C. µM	L.E.C. rank
	Top test conc. (µg/ml)	Top test conc. (mM)	Test concentration limitation		L.E.C. (µg/ml)	L.E.C. (µM)								
(i) Non-carcinogens with negative <i>in vivo</i> genotoxicity data														
Ampicillin trihydrate ^{*,§}	3710.00	10.00	mM	N	–		3710.00	10.00	mM	N	–			
D-Mannitol ^{*,§}	1822.00	10.00	mM	N	–		1822.00	10.00	mM	N	–			
(ii) Non-carcinogens with no <i>in vivo</i> genotoxicity data														
Phenformin HCl ^{*,§}	242.00	1.00	Cyto	N	–		242.00	1.00	Cyto	N	–			
n-Butyl chloride	926.00	10.00	mM	N	–		926.00	10.00	mM	N	–			
(2-Chloroethyl)trimethyl- ammonium chloride	24.60	0.16	Sol	N	–		24.60	0.16	Sol	N	–			
Cyclohexanone ^{*,§}	982.00	10.00	mM	N	–		982.00	10.00	mM	N	–			
N,N-dicyclohexyl thiourea	343.00	1.43	Sol	N	–		300.00	1.25	Sol	N	–			
Trisodium EDTA [*]	3582.00	10.00	mM	N	–		^a 4122.33	10.00	mM	P	2061.00	5000	5000.00	20
Ephedrine sulfate [*]	2152.20	5.02	Cyto	N	–		Not tested							
Erythromycin stearate	125.00	0.10	Sol	N	–		530.70	0.50	Sol	N	–			
Flumetron	581.00	2.50	Sol	N	–		581.00	2.50	Sol	N	–			
Phenanthrene	223.00	1.25	Sol	N	–		223.00	1.25	Sol	N + –	–			
(iii) Non-genotoxic carcinogens														
D-Limonene	1362.00	10.00	mM	N	–		1362.00	10.00	mM	N	–			
Di-(2-ethylhexyl)phthalate	488.25	1.25	Sol	N	–		3905.60	10.00	mM	N	–			
Amitrole [*]	840.00	10.00	mM	N	–		841.00	10.00	mM	N	–			
Tert-butyl alcohol	741.00	10.00	mM	N	–		741.00	10.00	mM	N	–			
Diethanolamine	1051.00	10.00	mM	N	–		1051.00	10.00	mM	N + –	–			
Melamine	1261.00	10.00	mM	N	–		1261.00	10.00	mM	N	–			
Methyl carbamate	751.00	10.00	mM	N	–		751.00	10.00	mM	N	–			
Progesterone	200.00	0.64	Sol	N	–		200.00	0.64	Sol	N	–			
Pyridine ^{*,§}	800.00	10.10	mM	N	–		791.00	10.00	mM	N	–			
Tris(2-ethylhexyl)phosphate	4347.00	10.00	mM	N	–		4347.00	10.00	mM	N	–			
Hexachloroethane	296.00	1.25	Sol	N	–		296.00	1.25	Sol	N	–			

^a Trihydrate salt.

^{*} Data from testing without S9 extracted from [7].

[§] Data from testing with S9 extracted from [19].

Table 3

Non-DNA-reactive chemicals (including non-genotoxic carcinogens), metabolic poisons and other substances that 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 toxicity.

Chemical	Test results without S9			GADD45a-GFP genotoxicity	Test results with S9		Top test conc. (µg/ml)	Top test conc. (mM)	Test con- centration limitation	GADD45a-GFP genotoxicity	L.E.C. (µg/ml)	L.E.C (µM)	Lowest L.E.C. (µM)	L.E.C rank
	Top test conc. (µg/ml)	Top test conc. (mM)	Test con- centration limitation		L.E.C. (µg/ml)	L.E.C. (µM)								
(i) Non-carcinogens that are negative or equivocal for genotoxicity <i>in vivo</i>														
D,L-Menthol	1563.00	10.00	mM	N	–	–	1563.00	10.00	mM	N	–	–	–	–
Phthalic anhydride*.§	1480.00	10.00	mM	N	–	–	740.00	5.00	mM	N	–	–	–	–
Tertiary-butylhydroquinone	19.50	0.12	AF	N	–	–	831.00	5.00	Cyto	P	51.94	312.52	156.26	14
o-Anthranilic acid	1371.00	10.00	mM	N	–	–	1371.00	10.00	mM	N	–	–	–	–
1,3-Dihydroxybenzene	1101.00	10.00	mM	N	–	–	1101.00	10.00	mM	N	–	–	–	–
2-Ethyl-1,3-hexanediol	1462.00	10.00	mM	N	–	–	1462.00	10.00	mM	N	–	–	–	–
Sulfisoxazole	1340.00	5.00	Sol	N	–	–	1340.00	5.00	Sol	N	–	–	–	–
(ii) Non-carcinogens with no <i>in vivo</i> genotoxicity data														
Ethionamide*	830.00	5.00	Sol	N	–	–	415.00	2.50	Sol	N	–	–	–	–
Curcumin	62.50	0.17	AF	U	–	–	115.13	0.31	Cyto	U	–	–	–	–
Benzyl alcohol	1081.00	10.00	mM	N	–	–	1081.00	10.00	mM	N	–	–	–	–
Urea	601.00	10.00	mM	N	–	–	601.00	10.00	mM	N	–	–	–	–
(iii) Non-genotoxic carcinogens or carcinogenic by irrelevant (for human) mechanism														
Sodium saccharin	2052.00	10.00	mM	N	–	–	2052.00	10.00	mM	N	–	–	–	–
(iv) Supplementary list (prediction of <i>in vitro</i> genotoxicity tests less clear)														
Propyl gallate	1061.00	5.00	Sol	P ++ –	132.63	625.02	1061.00	5.00	Sol	P	132.63	625.02	625	20
p-Nitrophenol	1391.00	10.00	mM	N	–	–	1391.00	10.00	mM	P	1391.00	10000.00	10,000	22
Sodium xylene sulfonate	2082.00	10.00	mM	N	–	–	2082.10	10.00	mM	N	–	–	–	–
Ethyl acrylate (not tested)														
Eugenol	411.00	2.50	Sol	N	–	–	411.00	2.50	Sol	N + – –	–	–	–	–
Isobutyraldehyde	721.00	10.00	mM	N	–	–	721.00	10.00	mM	N	–	–	–	–
2,4-Dichlorophenol*.§	324.00	2.00	Cyto	N	–	–	324.00	2.00	Cyto	P	81.00	500.00	500	18

* Data from testing without S9 extracted from [7].

§ Data from testing with S9 extracted from [19].

pond is limited due to the dilution by the sheath fluid that carries and aligns cells for analysis in the flow cell.

2.3. Compound dose and data acceptability criteria

The top dose tested was set at the current ICH S2B limit (10 mM, 5 mg/ml), and where this was achieved the test concentration limitation was recorded as “mM” in the tables. If the limit of solubility was lower than the ICH S2B limit, the test concentration limitation was recorded as “Sol” in the tables. For some samples colour or autofluorescence limited the top dose (“AF”) and these cases are discussed in Section 3. One compound (curcumin, Table 3) is labelled ‘U’ to signify that the data for this compound could not be interpreted due to the effects of intense autofluorescence. Acceptance of assay data are also dependent upon the degree of toxicity, which may arise at high concentrations of a test substance. In cases where an initial test with the highest concentration did not yield interpretable data because of excessive toxicity, the sample was retested starting from a lower top concentration that generated at least one dilution where there was a non-toxic (no-effect) dose: in such cases the test concentration limitation is recorded as “cyto” in the tables. In the spectrophotometric assay, data were rejected where the toxicity of the test substance limited the final cell density to 30% relative to the vehicle-treated controls during a 48-h exposure [7]. In the assessment of results from the flow cytometry test with S9, data were rejected when the minimum relative population survival, assessed by means of propidium-iodide fluorescence, was 20% compared with the vehicle-treated control [19]. This corresponds to the maximum level of toxicity at 80% used for other *in vitro* mammalian assays. S9 experiments have a shorter exposure time because of the increasing time-dependent toxicity of S9 itself. One consequence is that compounds can often be tested at a higher concentration with S9 than without S9—for some chemical concentrations, the cells can survive and recover from a 3-h exposure, but not a 48-h exposure.

Tables 1–3 correspond to the three tables defined by the ECVAM expert panel [6]. These in turn refer to the three groups of compounds recommended for testing. The tables provide summaries of results. All compounds were tested to the highest concentrations noted in the table and according to the criteria above. The compounds were prepared and tested by a panel of four operators. Three replicates for each compound were tested on different micro-plates and the compounds were assayed both with and without S9, producing at least six data points per compound. This allows reproducibility of the tests to be considered in data interpretation. The results of individual experiments are reported by a ‘P’ (positive) or an ‘N’ (negative), where triplicate assays produced three positive or three negative results, respectively. In other cases reproducible results are recorded as either ‘P + + –’ or ‘N + – –’.

Potency, defined here as the lowest effective concentration (L.E.C.) in a given test, is provided for compounds producing positive results. Where a compound is positive both with and without S9, the lowest L.E.C. (in μM) is used in ranking compounds according to potency.

3. Results

3.1. GreenScreen HC results for Group 1 compounds: *in vivo* genotoxins that should be detected as positive in *in vitro* mammalian cell genotoxicity tests

Table 1 provides the results for the Group 1 compounds—the expected positives. Of the 20 compounds in this group, 18 produced positive results, with and/or without S9, thus the sensitivity of the GreenScreen HC assay in this collection is 90%.

Table 1, section I lists the Ames-positive *in vivo* genotoxins. In subgroup (i), the O⁶ and N7 alkylators all came out positive. In subgroup (ii), the polycyclic aromatic hydrocarbons all gave positive results. In subgroup (iii), the aromatic amines were all positive, including the highly fluorescent 2,4-diaminotoluene (2,4-DAT), for which data were collected with flow cytometry in tests with and without S9. In subgroup (iv), the ‘others’ gave positive test results, with the exception of *p*-chloroaniline, which produced only negative results.

Table 1, section II shows results for the *in vivo* genotoxins that are negative or equivocal in the Ames test. These chemicals gave positive test results, with the exception of chloramphenicol, which produced only negative results.

Eight compounds in Table 1 (Group 1) were noted in the ECVAM paper as requiring metabolic activation, seven of these came out positive with S9 in this study. The exception was 2,4-DAT, which gave a negative response with S9, but was positive without S9.

3.2. GreenScreen HC results for Group 2 compounds: *non-DNA-reactive chemicals (including non-genotoxic carcinogens) that should give negative results in *in vitro* mammalian cell genotoxicity tests*

Table 2 provides the results for the Group 2 compounds—the expected negatives. Of the 23 compounds in this group, 22 produced negative results. Thus the specificity of the GreenScreen HC assay in this collection is 96% (22/23). In subgroup (i), the non-carcinogens with negative *in vivo* genotoxicity data both came out negative. In subgroup (ii), the non-carcinogens with no *in vivo* genotoxicity data all gave negative test results, with the exception of trisodium EDTA trihydrate, which only produced positive results at the highest tolerated dose (5 mM) with S9. Phenanthrene gave a single positive result with S9, but this could not be reproduced. In subgroup (iii), the non-genotoxic carcinogens all came out negative. Diethanolamine gave a single irreproducible positive result with S9.

3.3. GreenScreen HC results for Group 3 compounds: *non-DNA-reactive chemicals (including non-genotoxic carcinogens), metabolic poisons and other substances that should give negative results in *in vitro* mammalian cell genotoxicity tests, but have been reported to produce positive results in *in vitro* mammalian genotoxicity tests at high concentrations or at high levels of cytotoxicity*

Table 3 provides the results for the Group 3 compounds. Of the 19 compounds in this group, ethyl acrylate was not tested due to its volatility, and the intense colour and autofluorescence of curcumin made the data uninterpretable. Of the 17 compounds tested, 13 produced negative results and four gave positive results. In subgroup (i), the non-carcinogens that are negative or equivocal for genotoxicity *in vivo* came out negative, with the exception of tertiary-butylhydroquinone (TBHQ), which was negative without S9 but reproducibly positive with S9. In subgroup (ii), the non-carcinogens with no *in vivo* genotoxicity data all showed negative test results. The only compound in subgroup (iii), saccharin, was negative in the GreenScreen HC assay. In subgroup (iv), a supplementary list of compounds where the predictions of other *in vitro* genotoxicity results are less clear, three compounds were negative (eugenol produced a single irreproducible positive, only with S9) and three were positive: propyl gallate was reproducibly positive both with and without S9, whereas *p*-nitrophenol and 2,4-DCP were reproducibly positive only with S9.

4. Discussion

The expert group [6] noted that the similar numbers of carcinogens/genotoxins and non-carcinogens/non-genotoxins in Tables 1 and 2, respectively, provide a good balance for concordance calculations. For the GreenScreen HC assay, the figures for sensitivity (18/20 = 90%) from Group 1 (Table 1) and for specificity (22/23 = 96%) from Group 2 (Table 2) produced a concordance figure of 93%. Inclusion of figures from Group 3 (Table 3: 13/17) which includes less clear predictions of genotoxicity, distorts this balance, but the overall agreement with expected results remains high (53/60 = 88%).

As noted above, ethyl acrylate was not tested due to its volatility, and ephedrine was not available for testing with S9. Thus 61 compounds were tested three times without S9 and 60 compounds were tested with S9. Of the 121 resulting data sets 116 (96%) produced the same results three times (three positive results or three negative results). It appears that the additional handling steps used for the S9 studies reduced the reproducibility only

marginally: 57/60 (95%) produced the same result three times and three compounds were judged negative by the predominance of negative responses in the three separate tests. Without S9, 59 of 61 (97%) compounds produced the same result three times, with just one compound judged positive by the predominance of positive responses in the three separate tests, and one producing non-interpretable (U) data due to strong autofluorescence (curcumin, Table 3).

As noted in Section 1, these results were mostly produced on the basis of two fixed protocols. For compounds tested without S9, spectrophotometry was used for data collection; for compounds tested with S9, data were collected using flow cytometry. Fluorescent compounds were tested by use of flow cytometry, both with and without S9. In Group 1 (see Table 1), 2,4-DAT is highly coloured, so it was assessed using flow cytometry both with and without S9. The ECVAM expert group [6] noted that 2,4-DAT, an aromatic amide, requires metabolic activation. However, the US Environmental Protection Agency Gene-Tox program recorded positive mouse lymphoma *Tk*+/- assay results for 2,4-DAT in the absence of S9, and negative results in the presence of induced S9 [27]. Both the NTP and US National Library of Medicine 'TOXNET' databases [28] record results suggesting that 2,4-DAT is able to cause chromosomal aberrations regardless of the inclusion of S9. Thus the positive result for 2,4-DAT in the GreenScreen HC assay without S9 is perhaps not surprising.

In Group 1 (Table 1), section I, subgroup (iv), the aromatic amine *p*-chloroaniline produced only negative results in the GreenScreen HC assay. It is not clear why this chemical did not produce positive results: it is not a carcinogen, but produces positive results in *in vivo* comet assays, and variable results in mouse lymphoma and chromosome aberration assays [6]. In Group 1 (Table 1), section II (genotoxins, negative or equivocal in Ames), chloramphenicol produced only negative results in the GreenScreen HC assay, but it is not clear why. For this compound, the expert group [6] recorded inadequate carcinogenicity data; it produces a weak genotoxic effect at high doses (>1 mM) in rodent and human cells, and in V79 cells [29].

In Group 2 (Table 2: expected negatives), 22 of 23 compounds produced only negative results in the GreenScreen HC assay. The exception was trisodium EDTA trihydrate, which was only positive at the extreme dose (L.E.C. = 5 mM) and in the presence of S9. The expert panel noted that its genotoxicity in the mouse lymphoma assay is also associated with high dose. EDTA is a chelating agent, and its toxic effects are considered to be related to metal-ion deficiencies. Positive results have been reported in the *in vitro* comet assay (in L5178Y mouse lymphoma cells) for EDTA, as well as for nitrilotriacetic acid, another chelating agent, and also in the millimolar range [30]. In the latter study the presence of equimolar Ca^{2+} reversed the genotoxicity.

In Group 3 (Table 3: should be negative but often produce positive results) 13 of 17 compounds gave negative results. Of the four that gave positive results, two are antioxidants of the hydroquinone class: tertiary butyl hydroquinone (L.E.C. 156.26 μM ; subgroup i), and propyl gallate (L.E.C. 625 μM ; subgroup iv). Curcumin is also a member of this class of compounds but it produced non-interpretable results as a consequence of its intense colour and autofluorescence, even when flow cytometry was used. This is not a class of compounds that had been studied previously in the GreenScreen HC assay. There is increasing evidence that antioxidants can become pro-oxidants in the presence of oxygen and/or iron/copper ions [31–33]. It is plausible, therefore, that oxidative damage, as a consequence of these transformations, provides a common mechanistic explanation for the genotoxicity of these two compounds in the GreenScreen HC assay, so they are considered here together.

Tertiary butylhydroquinone (or 2-tertiary-butyl-4-hydroquinone; TBHQ) was reproducibly positive with S9. It is

a commonly used preservative for edible oils and fats (food additive E319). It is a primary antioxidant, acting as a free-radical scavenger. Anti-oxidative activity is associated with the donation of an electron or hydrogen atom to a peroxy radical. TBHQ, and its metabolite 2-*tert*-butyl-1,4-benzoquinone (TBQ), are toxic to human U937 cells and disrupt mitochondrial transmembrane potential [34]. Of relevance to the GreenScreen HC assay (+S9) results here, it has been reported that rat-liver microsomes can lead to the formation of the *tert*-butylsemiquinone anion radical from both TBHQ and from TBQ, which leads to semiquinone-dependent formation of superoxide ions [35]. These reactions most likely account both for TBHQ-induced DNA breakage *in vitro* [36], and for the formation of 8-hydroxydeoxyguanosine in calf-thymus DNA [37]. It is possible, therefore, that the positive result in the GreenScreen HC assay is a consequence of oxidative damage. Curcumin, also an antioxidant, is the principal active ingredient of the spice turmeric and it is present in many other foods. It appears to be protective against oxidative damage at low concentrations but genotoxic at higher concentrations [38]. *In vitro* studies have demonstrated copper ion-dependent cleavage of DNA by curcumin [39], as well as curcumin-induced oxidative damage to lung cells at non-cytotoxic doses [40]. The pro-oxidative properties of propyl gallate, another antioxidant (food additive E310), were demonstrated by its promotion of DNA-damage induction by bleomycin [41].

A plausible explanation for the fact that these compounds do not produce positive results in *in vivo* genotoxicity and animal cancer studies comes from the consideration of the work from Halliwell's group [reviewed in 31]. The environment of cells in culture is hyperoxic (150 mm Hg of O_2), which contributes to a state of considerably increased oxidative stress compared with that in cells of tissues not exposed to air (1–10 mm Hg of O_2). This hyper-oxic state increases leakage of reactive oxygen species (ROS) from electron-transport chains in the mitochondria [reviewed in 31]. The addition of antioxidants to the culture has been presumed to deal with this increased stress, but in fact even supplemented culture media are relatively deficient in antioxidants compared with circulating body fluids, leading to a permanent state of oxidative stress for cells in culture. This is exacerbated by the instability of culture-media constituents [31], whose oxidation products include hydrogen peroxide, quinones and semiquinones, which can react with and deplete cellular glutathione thus reducing the effectiveness of the cellular antioxidant defence. Thus, whilst in the low-oxygen *in vivo* environment the level of reactive oxygen species is a controlled balance between ensuring the functioning of electron-transport/redox signaling and minimizing oxidative stress, in the high-oxygen *in vitro* environment, the capacity of cells to maintain this balance is compromised. In fact, under hyper-oxic conditions induced by hyperbaric oxygen this balance can also be compromised *in vivo*, which leads to DNA damage in human leukocytes, as measured in the comet assay. However, this damage appears to be repaired rapidly, and with no associated increased in micronuclei or *HPRT* mutation (reviewed in [42]). Oxidative damage is an important and frequent mechanism of indirect DNA damage, whose effects are limited by competency in xenobiotic metabolism, redox balance, and the effective DNA-repair mechanisms, principally base-excision repair. As a consequence, ROS-mediated genotoxic carcinogenicity is often considered to be thresholded when considering compound safety (reviewed in [43]).

For the antioxidant compounds in this study, the limitation to ROS protection in the GreenScreen HC assay may in part reflect the capacity of the TK6 host cells to synthesize glutathione. From limited published data, TK6 cells appear to have low glutathione-S-transferase activity [44] and a lower competence in glutathione homeostasis than, for example, CHO cells [45].

Subgroup (iv) of Table 3 is a supplementary list of seven compounds where the predictions of *in vitro* genotoxicity results are less clear. Three of these substances were positive. 2,4-DCP is a metabolite of 2,4-dichlorophenoxyacetic acid (2,4-D) a well-known selective herbicide that produces chromosome aberrations in mice [46]. It was reproducibly positive in the GreenScreen HC assay with S9 (L.E.C. 500 μ M). It is unclear why these *in vivo* genotoxic effects do not translate into cancer hazard, but it might reflect the failure of 2,4-DCP to accumulate in the body: it was the most abundant phenolic compound found in a UK river study [47]. *p*-Nitrophenol was reproducibly positive in the GreenScreen HC assay, only with S9, and at the top dose (10 mM).

The high-dose positive result for *p*-nitrophenol (Table 3) draws particular attention to the current discussions surrounding the selection of the top dose for genotoxicity testing in the *in vitro* mammalian assays. The concentration 10 mM is the current ICH S2B-recommended maximum dose. In newly proposed recommendations (ICH S2(R1)), driven by the need to avoid misleading positive results, a reduced top dose of 1 mM is proposed for the *in vitro* mammalian tests. Two other positive compounds in this study have L.E.C.s above 1 mM: azidothymidine (5 mM) from the expected positives in Table 1 and trisodium EDTA trihydrate (5 mM) from the expected negatives in Table 2. Thus if the new 1-mM limit were applied, sensitivity based on the compounds in Table 1 would decrease from 18/20 to 17/20 and specificity based on Table 2 compounds would increase from 22/23 to 23/23, leading to no net change in the overall concordance drawn from Groups 1 and 2. There is a slight increase in overall agreement to 55/60 (92%), when Group 3 compounds are included.

For compounds that gave positive results, the lowest effective concentration (in μ M) was tabulated to provide a measure of potency. Of the expected positives in Table 1, sodium arsenite ranked highest (L.E.C. 0.009 μ M), and was over half a million times as potent as the lowest ranked compound, azidothymidine (L.E.C. 5 mM). The most potent of the genotoxins, active in the sub-micromolar range were all from Group 1: sodium arsenite, taxol, etoposide and aflatoxin B₁. The least potent genotoxins, active at 0.5 mM or above included azidothymidine and dimethylnitrosamine from Group 1, trisodium EDTA from Group 2 and propyl gallate and 2,4-dichlorophenol from Group 3. The L.E.C. is a valuable measure of potency, particularly for the pharmaceutical industry, where dose and exposure are critical in the assessment of safety.

These recommended lists have not yet been widely used, but a recent paper reported results for the assessment of the bacterial VitotoxTM and yeast RadarScreen assays [48]. Results are not directly comparable, because the compounds were all tested at lower top concentrations: Vitotox (0.1 mM); RadarScreen (1 mM). Thus the extreme conditions believed to contribute to poor specificity for Group 3 compounds were not assessed. Sensitivity, calculated from Group 1 was 70% (14/20) for both Vitotox and RadarScreen—a little lower than the figure for Greenscreen HC: 85% (17/20). The Vitotox and RadarScreen data from Tables 2 and 3 were combined to provide 'specificity' figures, and these were 93% (39/42) for Vitotox and 83% (35/42) for RadarScreen. Combining data from Tables 2 and 3 for GreenScreen HC and making a direct comparison possible by using data collected up to 1 mM, produces a 'specificity' figure for GreenScreen HC of 37/40 (93%).

5. Conclusions

As a follow-up to an ECVAM workshop, an expert group compiled a list of recommended compounds for the evaluation of new and improved *in vitro* genotoxicity tests [6]. In this study, compounds from this list were assessed in the GreenScreen HC assay,

providing a data set to allow comparisons to be made between the GreenScreen HC assay and other *in vitro* genotoxicity assays. The results from this study confirm findings from earlier published studies [7,19,22], which demonstrate that the GreenScreen HC assay exhibits sensitivity for genotoxins comparable with other *in vitro* mammalian cell assays, whilst at the same time maintaining a very high specificity.

Conflict of interest

I am a full time employee of Gentronix Ltd., the company which developed and sells the GreenScreen HC assay.

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