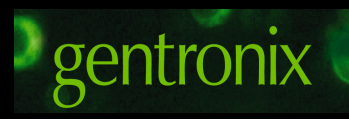


GADD45A-GFP TEST RESULTS FROM THE ECVAM RECOMMENDED LISTS OF COMPOUNDS FOR THE ASSESSMENT OF NEW OR IMPROVED GENOTOXICITY TESTS

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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 animal tests. It was anticipated that modified versions of existing assays as well as new assays might contribute to a solution, so an expert panel was asked to identify a list of chemicals that could be used in the evaluation of such assays [1]. Three categories of test chemical lists were chosen, and this manuscript provides test results from these chemicals using the GADD45a-GFP 'GreenScreen HC' assay. All were tested in triplicate, with and without S9, using invariant protocols.

Group 1 Chemicals should be detected as positive in *in vitro* mammalian cell genotoxicity tests:
Sensitivity: 18/20 (90%) were reproducibly positive in GreenScreen HC.

Group 2 Chemicals should give negative results in *in vitro* genotoxicity tests:
Specificity: 22/23 (96%) were reproducibly negative in GreenScreen HC.
Overall concordance from groups 1 and 2 was 93%.

Group 3 Chemicals should give negative results but can induce chromosomal aberrations or tk mutations often at high concentrations or at high levels of cytotoxicity:
 13/17 (76%) were reproducibly negative in GreenScreen HC.

Methods. All compounds were tested at 9 dilutions in a standardized 96-well format [2]. Data from S9 treated samples were collected using flow cytometry [3], and data from samples not treated with S9 were collected either using fluorescence/absorbance spectrometry [2] or flow cytometry. The two methods give the same results for compounds not requiring S9. Compounds were tested to 10mM, or lower if limited by solubility or toxicity. They were dissolved in a final DMSO concentration of 1% (v/v water), except for sodium arsenite (water alone) where DMSO is a confounding chemical factor for genotoxicity. All compounds have been tested at least 3 times both with and without S9. All compounds were tested using a final concentration 1% S9 derived from Aroclor 1254-treated rats.

Group 1 Results

Chemical	Test results without S9				Test results with S9				Lowest L.E.C. (µM)	L.E.C. Rank		
	Top Test Conc. (µg/ml)	Top Test Conc. (mM)	Test Limitation	GADD45a-GFP Genotoxicity	Top Test Conc. (µg/ml)	Top Test Conc. (mM)	Test Limitation	GADD45a-GFP Genotoxicity				
L Ames-positive <i>in vivo</i> genotoxins												
(i) O⁶ and N⁷ alkylators												
Cytophosphamide	25.00	0.09	Cyto	N	25.00	0.09	Cyto	P	6.25	22.39	10	
EN3 (N-Nitroso-N-ethyl urea)	2500.00	10.00	mM	P	1171.00	10.00	mM	P	36.80	312.50	17	
MMS	50.00	0.45	Cyto	P	50.00	0.45	Cyto	P	6.25	56.25	11	
(ii) Polycyclic aromatic hydrocarbons												
Benzo[a]pyrene	20.00	0.08	Sol	N	20.00	0.08	Sol	P	1.25	4.96	4.96	7
7,12-Dimethylbenz[<i>a</i>]anthracene	20.00	0.08	Sol	P	20.00	0.08	Sol	P	1.25	4.88	4.88	6
(iii) Aromatic amines												
Dimethylrosaniline	741.00	10.00	mM	N	741.00	10.00	mM	P	46.31	624.97	624.97	19
2-Acetylaminofluorene	200.00	0.90	Sol	N	200.00	0.90	Sol	P	50.00	223.94	223.94	15
2,4-Diaminotoluene	250.00	1.20	Sol	P	250.00	1.20	Sol	P	30.00	258.75	258.75	16
BD	240.00	1.21	Sol	P	240.00	1.21	Sol	P	30.00	151.34	151.34	13
PhP	160.00	0.71	Sol	P	160.00	0.71	Sol	P	1.25	5.57	5.57	8
(iv) Others												
Aflatoxin B1	10.00	0.03	Cyto	P	10.00	0.03	Cyto	P	0.04	0.12	0.12	4
Cadmium chloride	9.10	0.04	Cyto	N	14.32	0.08	Cyto	P	1.79	9.76	9.76	9
Cisplatin	16.00	0.05	Cyto	P	375.10	1.25	Cyto	P	5.86	19.53	244.5	5
p-chloroaniline	1276.00	10.00	mM	N	1276.00	10.00	mM	N	-	-	-	-
ii) <i>In vivo</i> genotoxins negative or equivocal in Ames												
Etoposide	5.00	0.01	Cyto	P	8.00	0.01	Cyto	N	-	-	0.06	3
Hydroquinone	12.30	0.11	AF	N	1101.00	10.00	mM	P	8.60	78.11	78.11	12
Acetaminophen	2670.00	9.99	mM	P	2672.40	10.00	mM	N	-	-	5000.00	21
Sodium arsenite	20.00	0.03	Cyto	P	159.81	0.03	Cyto	N	-	-	0.01	1
Taxol	4.00	0.01	Cyto	P	0.02	0.00	Cyto	P	0.06	0.08	0.03	2
Chloramphenicol	3231.00	10.00	mM	N	3231.00	10.00	mM	N	-	-	-	-

Exceptions in Group 1.

The aromatic amine, **p-chloroaniline** produced only negative results in the GreenScreen HC assay. It is not a carcinogen and doesn't form adducts, but produces positive results in *in vivo* Comet assays, and variable results in mouse lymphoma and chromosome aberration assays.

Chloramphenicol produced only negative results in the GreenScreen HC assay. The expert group recorded inadequate carcinogenicity data, and it produces a weak genotoxic effect at high doses (>1 mM) in rodent and human cells, and V79 cells.

Exception in Group 2

Trisodium EDTA trihydrate 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 MLA 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 also been reported in the *in vitro* Comet assay (in L5178Y mouse lymphoma cells) for EDTA, as well as for nitrilotriacetic acid (NAA) another chelating agent, and also in the millimolar range. In the Comet assay, the presence of equimolar Ca²⁺ reversed the genotoxicity of both EDTA and NAA.

Group 2 Results

Chemical	Test results without S9				Test results with S9				Lowest L.E.C. (µM)	L.E.C. Rank		
	Top Test Conc. (µg/ml)	Top Test Conc. (mM)	Test Limitation	GADD45a-GFP Genotoxicity	Top Test Conc. (µg/ml)	Top Test Conc. (mM)	Test Limitation	GADD45a-GFP Genotoxicity				
(i) Non-carcinogens with negative <i>in vivo</i> genotoxicity data												
Ampicillin trihydrate	2710.00	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-Chloroethyltrimethylammonium 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	3962.00	10.00	mM	N	*4122.33	10.00	mM	P	2061.00	5000	5000.00	20
Erginate sulphate	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	-	-	-	-
Amibole	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	-	-	-	-
Dihexanamine	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	0.84	Sol	N	200.00	0.84	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	-	-	-	-

Group 3 Results

Chemical	Test results without S9				Test results with S9				Lowest L.E.C. (µM)	L.E.C. Rank		
	Top Test Conc. (µg/ml)	Top Test Conc. (mM)	Test Limitation	GADD45a-GFP Genotoxicity	Top Test Conc. (µg/ml)	Top Test Conc. (mM)	Test Limitation	GADD45a-GFP Genotoxicity				
(i) Non-carcinogens that are negative or equivocal for genotoxicity <i>in vivo</i>												
D,L-methiol	1563.00	10.00	mM	N	1563.00	10.00	mM	N	-	-	-	-
Phthalic anhydride	1482.00	10.00	mM	N	740.00	5.00	mM	N	-	-	-	-
Tertiarybutylhydroquinone	19.50	0.12	AF	N	831.00	5.00	Cyto	P	51.94	312.52	1562.6	14
o-anthranic 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	850.00	5.00	Sol	N	415.00	2.50	Sol	N	-	-	-	-
Cucurbitin	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) Supplemental list (prediction of <i>in vitro</i> genotoxicity tests less clear)												
Propyl gallate	1061.00	5.00	Sol	P	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	N	-	-	10,000.00	22
Sodium selenite sulfonate	2962.00	10.00	mM	N	2962.10	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

Exceptions in Group 3

p-Nitrophenol was reproducibly positive in the GreenScreen HC assay, only with S9, and at the extreme dose (10 mM).

2,4-Dichlorophenol was reproducibly positive in the GreenScreen HC assay with S9 at 500 µM. It produces chromosome aberrations in mice.

Tertiary butylhydroquinone (L.E.C. 156.26 µM) and **propyl gallate** (L.E.C. 625 µM) are antioxidant food preservatives of the hydroquinone class. Both induced the reporter strongly. Antioxidants can become pro-oxidants in the presence of oxygen. Addition of the antioxidants in the assay medium (α-tocopherol or glutathione) did not reverse induction of the reporter by these chemicals.

Conclusions

The predictive statistics from these lists are similar to those generated in other studies with the GreenScreen HC assay. It has high sensitivity, comparable with the current *in vitro* mammalian assays, but a very much higher specificity. Its routine use would produce fewer falsely predictive positive results in compound screening and so help in reducing subsequent animal tests.