Evaluating DNA Reactivity Using the Acellular Comet Assay

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ABSTRACT

The high occurrence of positive results in the current battery of *in vitro* genotoxicity tests and their poor concordance with *in vivo* carcinogenicity results have been a significant source of concern in safety testing. Among the suspected causes of the high frequency of positive *in vitro* results are a dependency of most assays on cellular division and the confounding effects of cytotoxicity, repair, cell line differences and multiple modes of action. To supplement the specificity of *in vitro* testing and minimize the consequentially required *in vivo* tests, the acellular comet assay can be used to provide DNA reactivity dose response curve data using minimal test compound, time and expense. More sensitive and specific than the classic *in vitro* comet assay, the acellular comet assay avoids those factors that confound live cell assays while providing a fast method for determining DNA reactivity and elucidating the possible mode of action (MoA) of a test compound. To demonstrate how the acellular comet assay may be used effectively, we present DNA reactivity dose response curves generated for multiple compounds each with a different mode of action and/or genotoxicity profile.

INTRODUCTION

Although industries depend heavily on *in vitro* genotoxicity tests for the screening and safety assessment of most chemicals, the high "false positive" rate of these assays is of great concern where decisions must be made with minimal or no *in vivo* follow up testing (1-6). Examples of *in vitro*-specific conditions that can contribute to the poor specificity or sensitivity of an assay are:

(a) Cytotoxicity
(b) Cell division/cell cycle arrest
(c) DNA repair
(d) Cell line differences
(e) Test compound insolubility

So to supplement existing genotoxicity tests, the ideal test would be able to determine the DNA reactivity of a test compound in the absence of the above listed factors. This could help to identify compounds that may have a MoA that would result in a no-effect-concentration (NOEC) for the reasons described below.

(i) The compound exerts its genotoxic effect via primary damage to a non-DNA target
(ii) The compound, its metabolites or degradation products induce direct DNA damage, but only at toxic concentrations and/or concentrations above a defined threshold that is not reached *in vivo* or in humans.
(iii) The compound induces damage by a process that is specific to the *in vitro* test system or conditions that will never occur *in vivo* or in humans

The acellular assay provides a fast and highly sensitive method for determining the DNA reactivity of test compounds by eliminating those cellular processes and requirements that limit the sensitivity and/or specificity of other *in vitro* genotoxicity tests. By directly exposing wild type human DNA—rather than live cells—to as many as 15-20 dose concentrations (nm to M) and in 2-5 replicate independent experiments, our protocol can evaluate the DNA reactivity of test compounds, their metabolites and even degradation products at concentrations and under conditions more relevant to human exposure. And unlike standard *in vitro* assays, the multiple independent acellular comet experiments can all be conducted and analyzed in just a few days thus providing informative DNA reactivity data with the use of only nominal amounts of chemical and time.

METHODS & MATERIALS

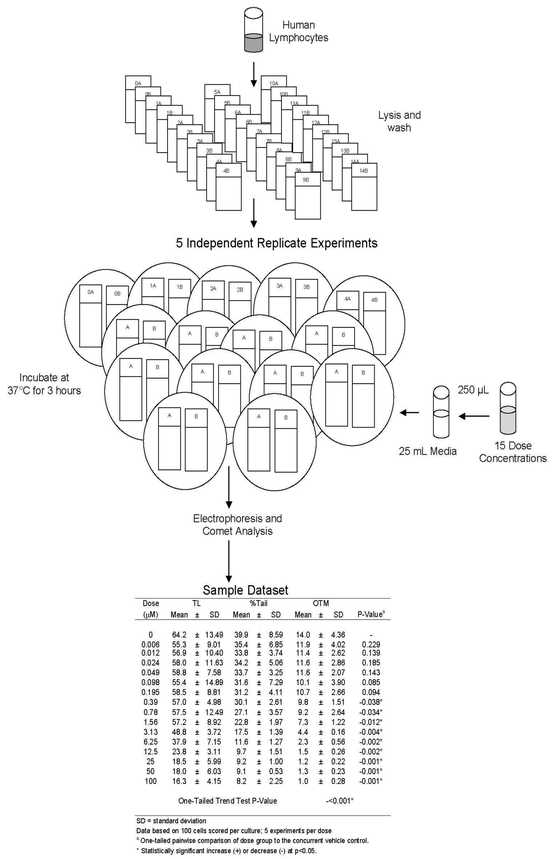
Test System

Whole blood was collected by venipuncture from a normal healthy non-smoking female volunteer. The whole blood was mixed sodium heparin anticoagulant. Immediately after collection, lymphocytes were isolated from the whole blood using Histopaque®1077 (Sigma). The untreated lymphocytes were mixed with mincing buffer (Mg^{++} and Ca^{++} free Hanks Balanced Salt Solution, 10% v/v DMSO and 20 mM Na_2EDTA, pH 7.4-7.7) at a $1x10^6$ mg/mL concentration before being flash frozen in liquid nitrogen and stored at –80±10°C for no more than 1 week before use.

Test Compounds

Allof the test compounds were obtained from Sigma Aldrich (St. Louis, MO). EMS, Cisplatin and Formaldehyde were prepared in dH2O. Chlorambucil, Aphidicolin, and Etoposide were prepared in DMSO.

Acellular Comet Assay



Sample Dataset

Dose (µM)	TL Mean	±	SD	%Tail Mean	±	SD	OTM Mean	±	SD	P-Value[a]
0	64.2	±	13.49	39.9	±	8.59	14.0	±	4.36	-
0.006	55.3	±	9.01	35.4	±	6.85	11.9	±	4.02	0.229
0.012	56.9	±	10.40	33.8	±	3.74	11.4	±	2.62	0.139
0.024	58.0	±	11.63	34.2	±	5.06	11.6	±	2.86	0.185
0.049	58.8	±	7.58	33.7	±	3.25	11.6	±	2.07	0.143
0.098	55.4	±	14.89	31.6	±	7.29	10.1	±	3.90	0.085
0.195	58.5	±	8.81	31.2	±	4.11	10.7	±	2.66	0.094
0.39	57.0	±	4.98	30.1	±	2.61	9.8	±	1.51	-0.038*
0.78	57.5	±	12.49	27.1	±	3.57	9.2	±	2.64	-0.034*
1.56	57.2	±	8.92	22.8	±	1.97	7.3	±	1.22	-0.012*
3.13	48.8	±	3.72	17.5	±	1.39	4.4	±	0.16	-0.004*
6.25	37.9	±	7.15	11.6	±	1.27	2.3	±	0.56	-0.002*
12.5	23.8	±	3.11	9.7	±	1.51	1.5	±	0.26	-0.002*
25	18.5	±	5.99	9.2	±	1.00	1.2	±	0.22	-0.001*
50	18.0	±	6.03	9.1	±	0.53	1.3	±	0.23	-0.001*
100	16.3	±	4.15	8.2	±	2.25	1.0	±	0.28	-0.001*
One-Tailed Trend Test P-Value										<0.001*

SD = standard deviation
Data based on 100 cells scored per culture; 5 experiments per dose
[a] One-tailed pairwise comparison of dose group to the concurrent vehicle control.
* Statistically significant increase (+) or decrease (-) at p<0.05.

METHODS & MATERIALS (CONT.)

Acellular Assay (Cont.)

Comet slides were prepared from the frozen human lymphocytes and lysed in low salt buffer (0.75M NaCl, 100 mM Na2EDTA, 10 mM Tris, pH 10, with 10% DMSO and 1% Triton X-100) to enable the expression of protein-DNA interactions (7). After lysis, duplicate slides of nuclear DNA were exposed to 15 dose concentrations of each compound in 5 independent Immediately after exposure, slides were exposed to alkali electrophoresis buffer (300 mM NaOH, 1 mM Na2EDTA; pH>13) for 20 minutes to unwind or denature the DNA. After unwinding, the slides were electrophoresed for 40 minutes at 0.7V/cm, 300±10 mA and 4°C, neutralized, and allowed to air dry. Air dried slides were stained with SYBR Gold™ stain (Molecular Probes) and 100 cells per dose (50 cells per slide) were scored using the Komet© GLP Image Analysis System. All slides were scored without knowledge of their identity. To provide statistical strength and comparability to *in vivo* comet assay data, the culture or dish (i.e. dose) in each independent experiment was treated as the unit of exposure.

RESULTS

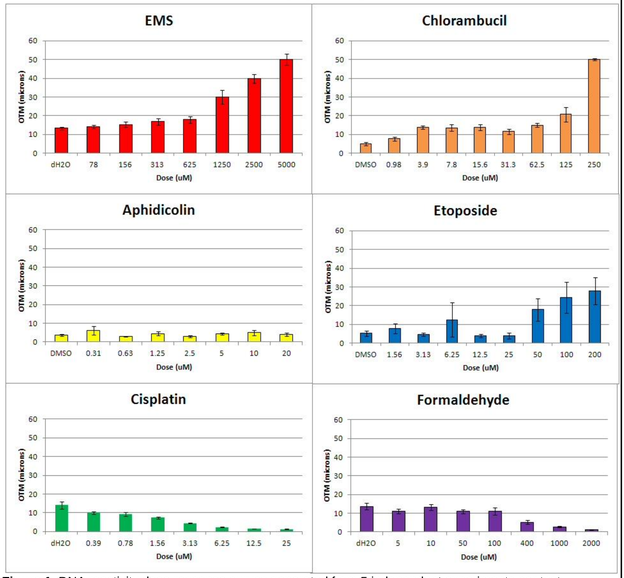


Figure 1. DNA reactivity dose response curves generated from 5 independent experiments per test compound: In the absence of cytotoxicity or cell division the following effects in DNA migration were detected after 3 hr of exposure : (a and b) EMS and Chlorambucil induced a dramatic dose response increase; (c) aphidicolin did not induce any effect; (d) etoposide induced a significant increase with high variability; (e and f) cisplatin and formaldehyde induced a dramatic dose response decrease.

As expected, the alkylating agent EMS induced a dramatic and dose-related increase in DNA migration while the DNA-DNA crosslinker cisplatin and the protein-DNA crosslinker formaldehyde induced dramatic and dose-related decreases. Also as expected, Aphidicolin did not have any direct effect on DNA migration as it indirectly induces strand breaks by inhibiting the completion of excision repair. Surprisingly, the topoisomerase II inhibitor Etoposide appeared to directly interact with DNA in the absence of cell division or metabolism. However, high inter-and intra-variability appears to indicate the presence of opposing mechanistic influences on DNA migration. Also a surprise, Chlorambucil induced extremely high levels of DNA strand breaks with no induction of crosslinks, one of its suspected modes of action. The dH_2O induced consistently higher migration than DMSO, most likey due to hydrolysis induced by the dH_2O.

RESULTS (CONT.)

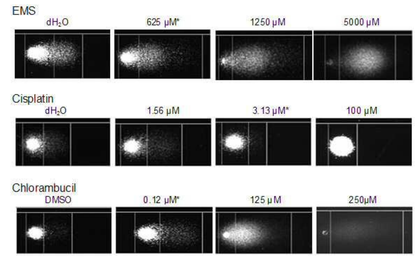


Fig. 2: Comet images captured with the Komet© GLP Image Analysis System provide visualization of the various comet shapes as they relate to the detection of strand breaks/alkali-labile sites (EMS and Chlorambucil) or crosslinks (Cisplatin and Formaldehyde).

Note: Despite claims that comets resembling "hedgehogs" (1250 and 5000 µM EMS) or "ghosts" (250µM Chlorambucil) are indicators of apoptosis or necrosis, these comets are exclusively due to genotoxicity as detected by the acellular comet assay. *=LOEL.

CONCLUSIONS

Our data demonstrate that the acellular comet assay using a single 3 hr sample time can provide reproducible information about the DNA reactivity of test compounds across a wide range of dose concentrations. And that the compounds evaluated in this experiment may be used as positive or comparative controls for determining the mode of action of other test compounds. Although this assay is limited and cannot be used to detect all forms of genotoxicity (e.g. aneuploidy), it may be used to detect early or previously undetected events such as crosslink induction or DNA strand breaks that can lead to aneuploidy, cell cycle inhibition and /or apoptosis or mutations. Since the acellular assay does not require the maintenance of live cells in culture, exposures can also include impurities or industrial chemicals that may require the use of cytotoxic solvents or vehicles. Or the studies may be designed to more closely mimic *in vivo* exposure conditions where cells can be exposed to dose suspensions in methyl cellulose (versus DMSO) or metabolites in the circulating plasma. This infinite flexibility combined with the sensitivity of comet provides a powerful investigative tool for understanding more about the mode of actions of many compounds. With it, we may have the ability to determine with greater confidence the true nature of "false positive" findings and better characterize what may be considered "well-characterized" compounds.

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