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Etoposide; colchicine; mitomycin C and cyclophosphamide tested in the in vitro mammalian cell micronucleus test (MNvit) in Chinese hamster lung (CHL) cells at Covance laboratories; Harrogate UK in support of OECD draft Test Guideline 487

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ABSTRACT

The following genotoxic chemicals were tested in the in vitro micronucleus assay, at Covance Laboratories, Harrogate, UK in the Chinese hamster lung cell line CHL. Etoposide (a topoisomerase inhibitor), colchicine (an aneugen), mitomycin C (a DNA cross linking agent) and cyclophosphamide (an alkylating agent requiring metabolic activation) were treated with and without cytokinesis block (by addition of cytochalasin B). This work formed part of a collaborative evaluation of the toxicity measures recommended in the draft OECD Test Guideline 487 for the in vitro micronucleus test. The toxicity measures used, detecting both cytostasis and cell death, were relative population doubling, relative increase in cell counts and relative cell counts for treatments in the absence of cytokinesis block, and replication index or cytokinesis blocked proliferation index in the presence of cytokinesis block. All of the chemicals tested gave significant increases in the percentage of micronucleated cells with and without cytokinesis block at concentrations giving approximately 60% toxicity (cytostasis and cell death) or less by all of the toxicity measures used.

The outcomes from this series of tests support the use of relative increase in cell counts and relative population doubling, as well as relative cell counts, as appropriate measures of cytotoxicity for the non-cytokinesis blocked in vitro micronucleus assay.

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1. Introduction

As detailed by Kirkland [1] the present study is part of an inter-laboratory comparative exercise to establish whether relative population doubling (RPD) and relative increase in cell count (RICC) are appropriate measures of cytotoxicity, and are capable of taking into account both cytostasis and cell death, in the in vitro micronucleus assay.

The well characterised genotoxic chemicals, etoposide (a topoisomerase inhibitor), colchicine (an aneugen), mitomycin C (a DNA cross linking agent) and cyclophosphamide (an alkylating agent requiring metabolic activation) were tested in the in vitro micronucleus assay, in the Chinese hamster lung cell line (CHL) at Covance Laboratories, Harrogate, UK.

Testing was performed in the presence and absence of cytokinesis block with replication index (RI) used as the toxicity measure in the presence, and relative cell count (RCC), RPD and RICC used in the absence of cytokinesis block.

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Testing methodology and assay criteria were as defined in OECD draft Test Guideline 487, for the in vitro mammalian cell micronucleus test [2].

2. Materials and methods

2.1. Chemicals

The test chemicals, unless otherwise stated, were purchased by Covance Laboratories Limited from Sigma–Aldrich UK and supplied to all trial participants. Mitomycin C (CAS no. 50-07-7) was dissolved in sterile water, whereas etoposide (CAS no. 33419-42-0), colchicine (CAS no. 64-86-8), and, cyclophosphamide (CAS no. 6055-19-12) were dissolved in dimethyl sulphoxide (DMSO).

2.2. Cell and culture conditions

The CHL cell line was obtained from the laboratory of Dr M Ishidate (Japan) and was maintained in tissue culture flasks containing McCoys 5a media (Gibco, Paisley, Scotland) supplemented with 10% FCS and 50 U/mL Penicillin/50 μg/mL Streptomycin. The modal chromosome number for the clone of CHL cells used was 25.

CHL cells were confirmed to be free from any mycoplasma infections prior to use.

Approximately 24 h prior to chemical treatment, CHL cells were seeded in to 6 well tissue culture plates (10 cm^3) at a concentration of 1×10^5 cells/mL in supplemented McCoys 5a media in a total volume of 3 mL per well. Plates were held in a humidified incubator with 5% CO_2 in air for the duration of the study.

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2.3. Metabolic activation

Cyclophosphamide was tested in the presence of Aroclor-induced rat liver S-9 (Molecular Toxicology Incorporated, USA) at a final concentration of 2% (v/v) homogenate, prepared as follows.

Glucose-6-phosphate (180 mg/mL), NADP (25 mg/mL), potassium chloride (KCl) (150 mM) and rat liver S-9 were mixed in the ratio 1:1:1:2. An aliquot of this mix was added to each cell culture to achieve a final concentration of 2%.

Etoposide, colchicine and mitomycin C were tested in the absence of any exogenous metabolising system.

2.4. Chemical treatment

Exponentially growing CHL cultures were treated for 3 h in either the presence or absence of 2% S9 mix. Multiple concentrations of each chemical were tested, in duplicate with and without cytochalasin B, and 4–6 concentrations were selected for scoring based on extent of cytotoxicity by each of the measures used. Solvent control cultures were treated in quadruplicate. For chemicals dissolved in DMSO (etoposide, colchicine and cyclophosphamide) the test chemical treatments did not exceed 1% (v/v) of the final culture volume. For mitomycin C, which was dissolved in sterile water, test chemical treatments did not exceed 10% (v/v) of the final culture volume. All experiments were performed in duplicate.

For the treatments in the presence of cytochalasin B, following the 3-h treatment phase, cultures were washed twice with 0.85% (w/v) sterile saline solution and 3 mL of supplemented McCoys 5a media, containing cytochalasin B at a final concentration of 3 μg/mL, was added. For cultures treated in the absence of cytochalasin B, the same procedure was followed with fresh media added without cytochalasin B. In both regimes, after the chemicals were washed off, cultures were incubated for approximately 21 h before harvesting.

2.5. Harvesting

Approximately 24 h after the start of treatment, culture media were removed and transferred into a 10 mL tube containing 5 mL supplemented McCoys 5a media, wells were washed once with 2 mL sterile 0.85% saline which was removed and retained, 1.5 mL trypsin EDTA (Invitrogen UK) was added to each well and incubated for a further 5–8 min until cell monolayers visibly detached. This cell suspension was then added to the previous washes and culture media, and 200 μL samples from cultures harvested in this way were added to 19.8 mL isoton (Beckman Coulter, UK) prior to counting on a Coulter counter (Beckman Coulter, UK). The remaining cell suspension was adjusted to a concentration of 6×10^4 cells/mL and centrifuged (1000 rpm for 5 min) on to clean labelled microscope slides using a Cytospin (Shandon, UK). After being allowed to slowly air dry, slides were fixed in ice cold 90% (v/v) methanol. Slides were stained with a solution containing 125 μg/mL acridine orange (Sigma, UK) for 10 s and rinsed with phosphate buffered saline, pH 7.4, for 10 min before being allowed to air dry in the dark.

2.6. Determination of cytotoxicity

Methods were as described by Lorge et al. [3] and Kirkland [1], briefly:

Replication index for cultures in the presence of cytochalasin B, was defined as:

$$\frac{\text{No. binucleated cells} + 2x\ \text{No. multinucleate cells/Total number of cells treated cultures}}{\text{No. binucleated } cells + 2x\ \text{No. multinucleate cells/Total number of cells control cultures}} \times 100$$

The following toxicity measures are all calculated from cultures in the absence of cytochalasin B:

Relative cell count was defined as:

$$\frac{\text{Final cell count in treated cultures}}{\text{Final cell count in control cultures}} \times 100$$

Relative increase in cell count was defined as:

$$\frac{\text{Increase in number of cells in treated cultures (final count} - \text{initial count)}}{\text{Increases in number of cells in control cultures (final count} - \text{initial count)}} \times 100$$

Relative population doubling was defined as:

$$\frac{\text{Number of Population doublings in treated cultures}}{\text{Number of Population doublings in control cultures}} \times 100$$

Table 1
Etoposide, 3-h treatments without S-9, toxicity and micronucleus data.

Dose (μg/mL)	Without Cyto B				With Cyto B	
	%Tox			%MN	%Tox	%MN
	RCC	RICC	RPD	Mo	RI	Bi
0	0	0	0	0.4	0	0.8
2	11	14	8	12.2***	1	NS
2.5	12	16	10	NS	15	27***
3	26	36	23	14***	16	NS
4	44	60	43	14.3***	38	35***
5	49	67	51	13***	50	36***
5.5	52	71	55	12.1***	64	NS

RCC, relative cell count; RICC, relative increase in cell counts; RPD, relative population doubling; RI, replication index; MN, micronucleate; Mo, mononucleate; Bi, binucleates; NS, not scored.
* Significant increase above control ($p < 0.05$).
** Significant increase above control ($p < 0.01$).
*** Significant increase above control ($p < 0.001$).

Table 2
Colchicine 3-h treatments without S-9, toxicity and micronucleus data.

Dose (μg/mL)	Without Cyto B				With Cyto B		
	%Tox			%MN	%Tox	%MN	
	RCC	RICC	RPD	Mo	RI	Bi	Mo
0	0	0	0	1.0	0	0.5	0.6
0.25	4	6	4	0.7	11	NS	
0.5	33	50	37	2*	18	1.2**	6.4***
0.75	43	66	53	3.7***	25	NS	
1.0	54	82	72	6.7***	29	1.9***	20.7***
1.25	60	92	86	4.3***	31	NS	
1.5	67	100	100	NS	52	2.1***	16.1***

* Significant increase above control ($p < 0.05$).
** Significant increase above control ($p < 0.01$).
*** Significant increase above control ($p < 0.001$).

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