Human Melanoma Cell line (A375): As a Potential In Vitro Micronucleus Assay Test System for Genotoxicity Assessment

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Introduction

The *in vitro* MN (MNvit) assay is a relatively simple, objective, and robust method commonly used in the evaluation of genotoxic potential of test compounds. Current testing guidelines (OECD 487) describe several cell lines suitable for use in the MNvit assay. Some cell lines, such as TK6, CHO, V79, or CHL, are limited by being either p53 deficient or inefficient and/or known to have significant apoptotic cells, which may interfere in the evaluation of some test compounds. Therefore, the present study was designed to evaluate the potential of the human melanoma cell line (A375) as an alternate test system, considering its relatively larger cytoplasmic area and lower frequency of apoptotic cells (less than 1%). This adherent cell line is p53 proficient and possesses epithelial morphology. Three well characterized compounds with varying mechanisms of action were evaluated in 5 independent trials using the MNvit assay. Exponentially growing A375 cells were treated with Mitomycin C (0.07 - 0.6 µM) or Cyclophosphamide (4.48 - 35.83 µM) for 3h in the absence or presence of S9, respectively; and Noscapine (5.56 - 44.5 µM) for 30h without S9. All cultures were harvested at 30h after initial treatment and both % micronucleated cells (MNC) and cytotoxicity (relative population doubling) were enumerated. Mean vehicle control %MNC was 3.18 ±0.41. The cytotoxicity ranged from 0 to 60%. All compounds showed statistically significant dose-responsive increases in %MNC (2- to 5-fold) when compared to concurrent vehicle control. These data demonstrate that the A375 cell line seems to be a suitable alternate for MNvit assay providing objective results.

Objective

The purpose of this study is to explore the usefulness of the A375 cell line (malignant melanoma cells of human origin) as a test system for the in vitro micronucleus assay (MNvit). Three reference test compounds with different modes of action (Mitomycin C, CAS -50-07-7: a direct acting clastogen, Cyclophosphamide, CAS - 50-18-0: a metabolically dependent clastogen, Noscapine, CAS - 912-60-7: an aneugen) were evaluated in this pilot project. The assay design was in line with the OECD testing guideline for in vitro micronucleus evaluation (OECD 487). The study was conducted using the Good Laboratory Practice (GLP) regulations for nonclinical laboratory studies as a guideline.

Methods

Test system and culture condition: A375 cells were obtained from the American Type Culture Collection (repository number CRL-1619^{TM}, Lot No. 70042943, Manassas, VA). A375 cells are derived from human malignant melanoma, adherent in nature and have epithelial- like morphology. Cells are hypotriploid with a modal number of 62 chromosomes and a population doubling time of 20h and harbor wild type p53 gene. The stock cell line was checked for stability of the modal chromosome number and was determined to be free from mycoplasma contamination. Cells were grown in complete medium (DMEM + 2 mM glutamine + 10% inactivated fetal bovine serum, 100 units penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B, and 20 mM HEPES) at a target of 5 x 10^5 cells/flask in vented T_{75} flasks. Cells were grown in standard tissue culture conditions (37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air) and were sub-cultured at 2-day intervals.

Experimental design:

T-25 cm^2 flasks seeded at (2.5mL/flask) @ target cell density of 1.6 x 10^5 cells/mL → 24h later, base line count and treatment initiated → Short term treatment 3h with and without activation followed by 27h recovery. Long term treatment 30h continuous without recovery → All cultures harvested at 30h from initiation of treatment

Treatment: Cells were seeded at 4 x 10^5 cells/flask in vented T-25 flasks 24 h prior to treatment. Phenobarbital/5,6 Benzoflavone induced liver homogenate S9 obtained from MolTox; Boone, NC and used at 1.5% v/v.

Mitomycin C (MMC) and Cyclophosphamide (CP) were tested in short term 3h exposure + 27h recovery, Noscapine (NOS) was tested in continuous 30h exposure + 0 h recovery.

Results and Discussion

Statistically significant induction in micronucleus frequency was observed for 3 reference test compounds.

Baseline frequency of micronucleus in solvent controls ranged from 2.6 to 5%.

Population doubling and micronucleus frequency were consistent among 5 independent trials up to cell passage number 25.

Micronucleus was clearly separated from the nucleus due to relatively larger cytoplasm.

Apoptotic cells were less frequent (≤ 2 /1000 cells).

Due to low apoptotic frequency and larger cytoplasmic: nucleus ratio in A375 cells, slide scoring was relatively faster.

The TK6 cell line that is frequently used in the micronucleus assay, has relatively higher frequency of apoptotic cells. When apoptotic cells rupture during culture processing, the nuclear fragments interfere in the enumeration of micronucleus that may lead to false positive results (Whitwell et al., 2015). These smaller cytoplasmic/nuclear rations of TK6 cells may appear as micronuclei close to nucleus, making it more challenging for the slide scorer to read, and therefore slowing down the scoring process. Our observation indicates A375 cells offer an advantage over the TK6 cells in the micronucleus assay.

Conclusions

The pilot study, with A375 cells showed it is equally sensitive as TK6 cells for MN induction. The larger cytoplasm and low apoptotic cell frequency make the assay more accurate and faster to score compared to TK6 cell-based micronucleus assay.

Reference

1. OECD Guideline for the Testing of Chemicals, Guideline 487 (*In Vitro* Mammalian Cell Micronucleus Test). Updated and adopted 29 July 2016.

2. Whitwell et al., Mutation Research, 789-790:7-27 2015.

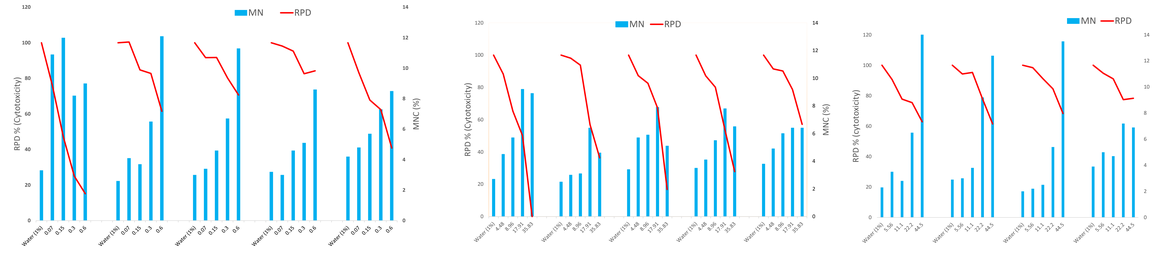

Figure 1: Concentration related increase in %- micronucleus frequency was observed for three reference test compounds with different mode of action (MMC= a direct acting clastogen; CP = metabolic activity required for clastogenic action; NOS= an aneugen). This indicates A375 cell is sensitive to detecting genotoxic compounds in in vitro micronucleus assays.

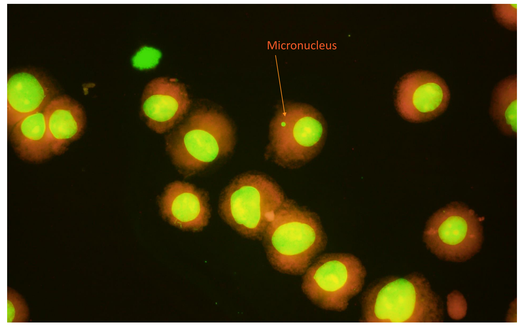

Figure 2: Microphotograph of A375 cells stained with acridine orange. The bigger cytoplasmic area and clear separation of micronucleus from main nucleus makes the microscopic slide scoring more efficient

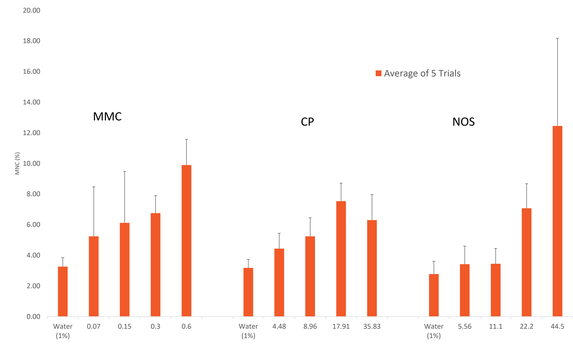

Figure 3: Average of % micronucleus frequency from 5 independent trials. Different cell passages (up to 25) were used in trials. Consistency in the background micronucleus frequency (3 ± 0.5) shows cell line has good genetic stability in the culture for relatively longer period.

ID	CONC (%)	S9	TREATMENT TIME (h)		COUNT (N)	PD	MICRONUCLEUS INDUCTION (%)				95% CI
			Exposure	Recovery			Mean	SD	Min	Max	
WATER	1%	-S9	3	27	5	1.43	3.6	0.59	2.6	4.2	
WATER	1%	+S9	3	27	5	1.32	3.18	0.55	2.5	3.8	
WATER	1%	-S9	30	0	4	1.59	2.78	0.84	2.2	5	

ID	CONC (µM)	S9	TREATMENT TIME (h)		COUNT (N)	PD	MICRONUCLEUS INDUCTION (%)				FOLD (Mean)
			Exposure	Recovery			Mean	SD	Min	Max	
MMC	0.07	-S9	3	27	5	1.29	5.24	3.24	3	10.9	1.61
MMC	0.15	-S9	3	27	5	1.12	6.12	3.36	3.7	12	1.87
MMC	0.3	-S9	3	27	5	0.97	6.76	1.14	5.1	8.2	2.1
MMC	0.6	-S9	3	27	5	0.8	9.9	1.68	8.5	12.1	3.18
CP	4.48	+S9	3	27	5	1.19	4.44	1	3	5.7	1.41
CP	8.96	+S9	3	27	5	1.1	5.24	1.21	3.1	6	1.64
CP	17.91	+S9	3	27	5	0.83	7.54	1.18	6.4	9.2	2.44
CP	35.83	+S9	3	27	5	0.39	6.3	1.67	4.6	8.9	2.04
NOS	5.56	-S9	30	0	4	1.5	3.43	1.18	2.2	5	1.23
NOS	11.1	-S9	30	0	4	1.42	3.45	1	2.5	4.7	1.24
NOS	22.2	-S9	30	0	4	1.25	7.08	1.6	5.4	9.2	2.64
NOS	44.5	-S9	30	0	4	1.08	12.45	5.72	5	18.9	5.14

Figure 4: Data from individual trials for reference compounds. Data shows mean ± SD and minimum and maximum values of micronucleus induction with respect to concentration. Average values of population doubling (PD) and 95% control limit of fold induction in %MN induction is also indicated. Count (N) represents number of independent trials.