



## **A comparison of the *in vitro* Genotoxicity of Anticancer Drugs Melphalan and Mitoxantrone**

**Susmita Mishra\* and Ravi Prakash Mishra**

Department of Post- graduate Studies & Research in Biological Sciences, R.D. University, Jabalpur, India

**\*Corresponding author:**

Susmita Mishra, Ph.D.

Department of Post- graduate Studies & Research in Biological Sciences

R.D. University,

Jabalpur- 482001( M.P.)

India

Email: [sus\\_biochem@rediffmail.com](mailto:sus_biochem@rediffmail.com)

*Received: 24 December 2012; / Revised: 17 March 2013; / Accepted: 18 May 2013*

### **Abstract**

Melphalan and Mitoxantrone are anthracycline antibiotics used in cancer therapy. This research was carried out to investigate *in vitro* genotoxic effects of the two (Melphalan and Mitoxantrone) anti-cancer drugs on human lymphocytes using Micronucleus Test. The effect induced by Melphalan was more pronounced than by Mitoxantrone. There was a significant increase in the induction of DNA damage in the cells treated with Melphalan. Our results indicate that 10  $\mu$ M concentration of Melphalan induced formation of 1% of frequency of Micronucleated Lymphocytes (MNLs) while the same concentration of Mitoxantrone does not show any MNLs. The 70  $\mu$ M concentration of Melphalan and Mitoxantrone both on an average induced 5.8 % and 3.7% frequency of MNLs respectively. We concluded that the Melphalan and Mitoxantrone are able to induce genotoxic effects in human lymphocytes *in vitro* in a dose-dependent manner, therefore, in normal lymphocytes much lesser genotoxicity of Mitoxantrone compared to Melphalan should be taken into account in planning chemotherapeutic strategies.

**Keywords:** Genotoxicity, Melphalan, Mitoxantrone, Lymphocytes, Micronucleated Lymphocytes.

### **1. Introduction**

Genotoxicity of anticancer drugs to normal cells is one of the most serious problems of chemotherapy due to the possibility of inducing secondary malignancies [1]. Although a precise definition of “genotoxicity” is elusive, there is no

doubt that DNA damage plays an important role in most mechanisms underlying the action of anticancer drugs interacting with DNA. It is therefore an imperative task in chemotherapy to determine the DNA-damaging effect of these drugs on normal cells [2,3,4].

A number of medicines /drugs used in cancer treatment such as Cisplatin,

Cyclophosphamide, Vinblastine, Ularin, Tamoxifen, Melphalan, Mitoxantrone etc. cause Cytotoxicity and Genotoxicity, but most of the anticancer drugs target the enzyme systems in the cell cycle to block cell division [5,6]. Melphalan and Mitoxantrone are anthracycline antibiotics widely used in cancer therapy. Mitoxantrone (1, 4-dihydroxy-5, 8-bis-[(2-[(2-hydroxyethyl) amino]-9, 10-anthracenedione) is an anthracycline analogue with better antineoplastic activity and less toxicity than Melphalan. It is an anthracenedione anthracycline analog. All anthracyclines consist of an aglycone ring coupled with an aminosugar and they can produce a wide range of biological effects. The planar ring can intercalate between DNA base pairs and the aminosugar moiety can interact with negatively charged phosphate groups in the DNA major groove [7,8]. The intercalation can cause changes in the shape of DNA helix, interfering with transcription and replication. Anthracyclines can also inhibit the activity of topoisomerase II, an enzyme introducing double-strand breaks in DNA [9,10,11,12].

Micronucleus test is most frequently used to assess Micronuclei (MNI). MNIs are chromatin containing structures in cytoplasm surrounded by a membrane without any detectable link to the cell nucleus formed by the exclusion of whole chromosomes or chromatin fragments during cell division. Elevated frequency of MN could be related to an overall genetic instability [13].

The changes in the genetic material of an organism can be detected at specific levels by using various genotoxicity assay system like chromosomal aberration, sister chromatid exchange, micronucleus assay and comet assay having different end point. Therefore, Evaluation of many new anti-neoplastic substances for anti-tumor therapy requires careful examination of its genotoxic properties chosen *in vitro*, which has a potential to revolutionize drug toxicity to determine the tolerable & threshold levels [14].

In the present work we compared the DNA-damaging potential of Melphalan and Mitoxantrone in normal human blood lymphocytes using the micronucleus test.

## 2. Materials and Methods

### 2.1 Chemicals

The anti-cancer drugs Melphalan and Mitoxantrone were selected for present study due to their extensive clinical use in India. Both the drugs were purchased from (Jabalpur, MP, India).

**Melphalan** hydrochloride (trade name: Alpeen) IUPAC name 4-bis (2-chloroethyl aminophenyl) alanine. Molecular weight: 305.2.

**Mitoxantrone** is an anthracycline. IUPAC name 1, 4 dihydroxy 5, 8 bis [2-(2-hydroxy ethyl amino)] -anthracene 9, 10-dione. Molecular weight: 444.5.

### 2.2 Collection of blood samples and isolation of lymphocytes

Blood samples were collected from three groups i.e. A, B and C. Group A consists blood donors of age group 18-22, group B consist age group 23-27 and group C consist age group 28-32 years.

Fresh blood from the five healthy non-smoking donors from each group was collected with the help of a physician and transferred in the eppendorf tubes and used immediately for the experiment. Seven ml of diluted blood was taken and pure lymphocytes were isolated from the blood sample by the density gradient centrifugation technique. The lymphocytes layer at the interface of HiSep (Hi media) and diluted plasma was collected with a sterile cut tip micro pipette and PBS ( $\text{Ca}^{++}$   $\text{Mg}^{++}$  ion free) medium RPMI at 22 °C. The resulting cell suspension was centrifuged at 250 X g for 10 minutes. The supernatant was discarded. The cells were re-suspended in 2-3 times volume of medium RPMI and centrifuged at 1500 rpm for 10 minutes. The supernatant was discarded and cells were re-suspended in suitable volume in order to get  $10^6$  cells / ml for use in viability test, comet assay and micronucleus test. Lymphocytes were treated with different concentrations of each drug for three hours at room temperature. The experiment was approved by the Institutional research committee, Rani Durgavati University (RDVV), Jabalpur (Reg. No. RDDC/866/2010).

### 2.3 Preparation of drug solution

One molar solution of Melphalan and Mitoxantrone were prepared by dissolving the gram molecular weight of the respective anti-cancer drugs in double distilled water. Various aliquots from the stock solution were used throughout the experiment as indicated.

### 2.4 Cell treatment

Melphalan and Mitoxantrone were taken from their stock solutions (0.5 and 4 mM, respectively) in RPMI 1640 and added to the suspension of lymphocytes to give final concentrations in the range of 0.01–10 mM. To control cells only RPMI 1640 was added. To examine DNA damage, the lymphocytes were incubated with the chemicals for 3 h at 37°C. Each experiment included a positive control, which was hydrogen peroxide at 20 mM. H<sub>2</sub>O<sub>2</sub> produced pronounced DNA damage, which resulted micronuclei's.

### 2.5 Assessment of cell viability

Viability of lymphocytes was determined by Trypan blue exclusion analysis [15]. Lymphocytes were incubated with drug at the concentration range of 0.0–70 µM for 3 h at 37°C, washed and re-suspended in RPMI 1640. An equal volume of 0.4% Trypan blue reagent was added to the cell suspension and the percentage of viable cells was evaluated under a field microscope. The assay is based on principle that the viable cells are non permeable for the dye while the dead cells lost this membrane property and turned blue.

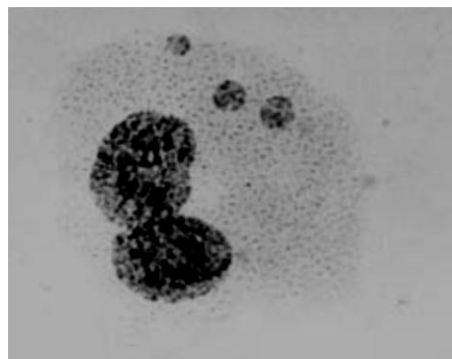
### 2.6 Micro-nucleus test to assess genotoxicity

The micro-nucleus test was performed according to the method described by Schmid, 1975 [16] to assess drug induced genotoxicity. The frequency of micro-nucleated lymphocytes (MNLs) was evaluated by examining 3000 mature lymphocytes for every replicates of each person and the percentage frequency was expressed as follows-

$$\text{Percentage frequency of MNLs (\%)} = \frac{\text{Total number of MNLs}}{\text{Total number of cells examined}} \times 100$$

### 2.7 Statistical analysis

Analysis of variance (ANOVA), mean and standard deviation (SD) were calculated to draw the inferences between drug treated and untreated samples.



**Figure 1.** Micro-nuclei in human lymphocytes treated with LC 50 dose of Mitoxantrone

## 3. Results and Discussion

All the anticancer drugs are highly cytotoxic agents and may be toxic to normal cell especially to rapidly dividing cells like bone marrow cells fetal cells, germ cells, hair follicle cells, intestinal cells, etc [17]. Anthracycline antibiotics like Malphalan and Mitoxantrone are widely used in cancer therapy. The ability to induce DNA damage in non-cancerous cells may underlie one of the most serious side effects evoked by anticancer drugs — induction of secondary malignancies [18]. In this study we aim to compare the responses of human lymphocytes to the anti cancer drugs Melphalan and Mitoxantrone. In order to evaluate genotoxicity of the drugs we have used Micronucleus Test. The tests used can determine not only the toxicity but also the dosage levels at which these can produce toxicity.

### 3.1 Cell viability

The results of cell viability tests after incubation of human lymphocytes with

Malphalan or Mitoxantrone at different concentrations show that both tested compounds caused a concentration-dependent decrease in cell viability (Figure 1). There were no significant differences ( $p > 0.05$ ) between the viability of the cells in the presence of either compound at concentrations up to 10  $\mu\text{M}$ , i.e. the maximal concentration of the drugs used in further experiments. Viability of the lymphocytes at 20

$\mu\text{M}$  drug concentration was about 81% with drug Melphalan, whereas, at the same concentration Mitoxantrone showed 95% viability (Table 1). Above 10  $\mu\text{M}$  drug concentration, Malphelon evoked a more pronounced decrease in viability of the lymphocytes than Mitoxantrone (upto 35% cells viable with Melphalan at 70  $\mu\text{M}$  vs. 50% for Mitoxantrone;  $p < 0.001$ ).

**Table 1.** *In vitro* viability of human lymphocytes in different concentrations of anti- cancer drugs

S.No.	Drug concentration ( $\mu\text{M}$ )	% Viability	
		Melphalan	Mitoxantrone
1.	0 (control)	100	100
2.	10	100	100
3.	20	81	95
4.	30	75	95
5.	40	50	80
6.	50	40	70
7.	60	35	60
8.	70	35	50

**Table 2.** Percentage frequencies of micro- nucleated lymphocytes in different concentration of drugs

SN	Drug Conc ( $\mu\text{M}$ )	Melaphalan (No. of MNs formed)			Mitoxantrone (No. of MNs formed)			% frequency of MNLs	
		R 1	R 2	R3	R1	R2	R3	Melaphalon	Mitoxantrone
1	0	0	0	0	0	0	0	0	0
2	10	10	8	12	0	0	0	1	0
3	20	18	20	19	12	15	18	1.3	0.9
4	30	28	30	25	16	18	20	1.8	1.3
5	40	40	35	32	22	28	27	2.1	1.7
6	50	52	58	56	31	35	32	2.9	2.1
7	60	68	65	69	48	46	43	4.1	2.9
8	70	81	78	82	59	55	3	5.8	3.7

## 3.2 Assessment of genotoxicity

### 3.2.1 Micro-nucleus Test

The numbers of micro-nucleated lymphocytes (MNLs) observed per 3000 cells examined at different concentrations of each drug are shown in Table 2. The micro-nucleus frequency in Melphalan treated lymphocytes vary from 1 to 5.8% and Mitoxantrone treated lymphocytes from 0 to 3.7%. This result indicates that Melphalan is more genotoxic as compared to Mitoxantrone.

### 3.2.2 Statistical analysis

Coefficient of variation for micronucleus formation was calculated. The coefficient of variation shows the comparative accuracy of the data with respect to both drug tested (Melphalan,  $y = 0.038x + 0.6$ ;  $R^2 = 0.989$  and for Mitoxantrone,  $y = 0.055x - 0.4$ ;  $R^2 = 0.3528$ ). It was found that Melphalan was more toxic than Mitoxantrone as concluded by statistical calculations. While comet pictures as observed in the microscope also indicate similar results as represented by the comet tail lengths versus the concentration of the drugs.

In the present study, we found that the 3.7% & 5.8% MN frequency at 70  $\mu$ m concentration of Mitoxantrone and Melphalan respectively detected by the micro nucleus test. To explain the results, in both drugs, Melphalan were found to have higher level of genotoxicity as compared to the level of genotoxicity observed in Mitoxantrone. Mitoxantrone (1, 4-dihydroxy – 5, 8-bis [(2-[(2-hydroxyethyl) amino] -9, 10-anthracenedione) is an anthracycline analog with better antineoplastic activity and less toxicity than Melphalan. Mitoxantrone inhibits topoisomerase II and displays a significantly reduced potential to form free radicals compared to anthracycline [19]. It is used for the treatment of breast cancer, prostate cancer, leukemia and lymphoma.

## Acknowledgements

We are thankful to Department of Biological Sciences, R.D. University, Jabalpur (M.P.) for providing laboratory facilities and also to the Head of the Department. Help received from Dr

Balwant Rawat and Dr Janhvi Mishra Rawat for the preparation of manuscript is greatly acknowledged.

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