

## <sup>12</sup>C-Beam Induces More Chromosomal Damage In Chemo-Radio-Resistant Cells Than <sup>16</sup>O-Beam

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### Brief Communication

Received date: 02/04/2018

Accepted date: 08/05/2018

Published date: 14/05/2018

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**Keywords:** Linear energy transfer, chromosomal aberrations, relative biological effectiveness, radio-resistance, chemo-resistance, radiotherapy.

#### ABSTRACT

The major challenge to eradicate cancer is the development of resistance to chemo- and/or radio-therapy. Recent studies indicate that particle radiation or high-LET (linear energy transfer) radiation kills cancer cells more effectively than low-LET gamma- or X-rays, potentially by inducing complex chromosomal aberrations (CAs). However, the efficacy of inducing chromosomal damage by various high-LET radiations has not been well-studied, specifically in cells resistant to both chemotherapeutic drug as well as ionizing radiation. To address the issue, we exposed a chemo-radio-resistant cell strain M5, which is derived from Chinese hamster lung fibroblast V79 cells, to <sup>12</sup>C- and <sup>16</sup>O-beams using a Pelletron accelerator. Aberration frequencies were scored at two different post-irradiation times (24 h and 48 h) using Giemsa staining technique. A linear dose-dependent increase in CAs was noticed at 24 h and 48 h after <sup>12</sup>C-irradiation. Similar linear dose-response was also observed after <sup>16</sup>O-irradiation at 24 h, but not at 48 h. In addition, difference in aberration spectrum was noticed based on radiation quality and post-irradiation incubation times. The dose-response curves revealed that <sup>12</sup>C-beam induces more CAs than <sup>16</sup>O-beam. This data clearly suggests that the extent and spectrum of chromosomal damage depends on the quality of high-LET radiation as well as the post-irradiation incubation time. These findings could potentially have applications in selecting beam parameters during hadron therapy (high-LET radiation therapy), particularly to eradicate chemo-radio-resistant tumors.

#### INTRODUCTION

Resistance to chemotherapeutic drug and/or therapeutic radiation continues to be a major obstacle for cancer treatment. Chemo-resistance may develop via increasing drug inactivation and/or efflux, enhancing DNA repair ability, inhibiting cell death, and promoting epithelial-mesenchymal transition (EMT) <sup>[1]</sup>. On the other hand, radio-resistant cancer stem cells may develop as a result of conventional radiotherapy with low-LET photons (X- and gamma-rays) because of EMT <sup>[2]</sup>. Therefore, development of an alternative therapeutic strategy is imperative to kill tumor cells effectively, especially those which have developed chemo-radio-

resistance.

As opposed to low-LET photons, particle radiations or high-LET radiations are more effective in cell killing because of their higher relative biological effectiveness (RBE) [3]. This higher RBE of particle radiation is thought to be the result of various factors, such as 1) deposition of more energy per unit distance, 2) generation of unreparable DNA damage 3) formation of complex chromosomal aberrations (CAs), and 4) similar cell-killing efficiency irrespective of cell cycle phases or cellular oxygen content [4]. Moreover, compared to low-LET radiations, high-LET radiations deposit energy more precisely, thus significantly reduces the risk of normal tissue toxicity surrounding the tumor [4]. All these beneficial properties indicate that high-LET radiation could be a better option as opposed to gamma- or X-rays in cancer treatment. However, it is first necessary to determine how the quality of various high-LET radiations influences the genotoxicity.

Ionizing radiation (IR) exerts genotoxic effects by inducing DNA double strand breaks (DSBs). Inappropriate rejoining of DSBs results in CAs, which subsequently may lead to induction of apoptosis [5]. We demonstrated that a strong correlation exists between cell death and the level of CAs/apoptosis after exposure to IR [6-8]. Importantly, nature of DNA damage, which depends on quality of radiation, influences the spectrum, yield, and complexity of CAs. For example, in contrary to low-LET radiations, high-LET radiations generate “dense” ionization events leading to “clustered” DNA damage [9]. These clustered damages are more prone to induce complex CAs. However, no study was undertaken to determine the effects of various high-LET radiations on CAs.

In this article we examine the spectrum of CAs in M5 cells, a chemotherapeutic drug (methotrexate) as well as to gamma-rays resistant cell strain [10,11], after exposure to various doses of <sup>12</sup>C-beam (LET = 295 keV/μm) and <sup>16</sup>O-beam (LET = 625 keV/μm) at different time intervals. We observed frequency and pattern of CAs depends on radiation quality and post-irradiation times.

## MATERIALS AND METHODS

### Cell line and cell culture

M5 cell strain was used in this study. The origin and characteristics of M5 have been previously described [10,11]. Cells were cultured in Eagle MEM medium (HiMedia, India); supplemented with 10% heat inactivated membrane sterilized Fetal Bovine Serum (Biological Industries, Israel), and 1% antibiotic, Gentamicin (Roche Diagnostics, GmbH).

### Irradiation

Irradiation procedure has been described elsewhere [8]. Briefly, 20 h prior to radiation exposure,  $0.6 \times 10^6$  cells were seeded in each of the specially fabricated Petri-dish like structure, composed of a steel ring measuring 24 mm in diameter with a 6 μ-thick polypropylene sheet. The confluent cell monolayer was exposed to <sup>12</sup>C- and <sup>16</sup>O-beam at Inter University Accelerator Centre (New Delhi, India) using a 15UD Pelletron accelerator as previously described [8]. Cells were exposed to various doses of <sup>12</sup>C-beam (0, 1.18, 2.36, and 4.73 Gy) and <sup>16</sup>O-beam (0, 2.46, 4.91, and 9.83 Gy), which correspond to identical particle fluence ( $0, 1 \times 10^6, 5 \times 10^6, \text{ and } 1 \times 10^7$  particle/cm<sup>2</sup>). The beam energy was calculated using the Monte Carlo Code TRIM. Homogeneity of the beam was measured with a surface barrier detector fitted with a collimator (aperture area 0.886 mm<sup>2</sup>). Immediately after irradiation, cells were briefly trypsinized, transferred to normal tissue culture Petri-dishes and incubated for 24 h and 48 h before harvest.

### Dosimetry

Dosimetry has been previously described [7]. Briefly, a silicon surface barrier detector was used to count the number of particles and also to measure the beam energy. Fluences was calculated from the relation of the detector count and aperture area of the collimator. Dose (Gy) was calculated from the quantities of fluence as follows:

$$\text{Dose [Gray]} = 1.6 \times 10^{-9} \times (dE/dx) [keV/\mu m] \times F [p/cm^2] \times 1/\rho [cm^3/gm]$$

Where  $dE/dx = \text{LET}$ ,  $\rho = \text{density of stopping material}$  (in this case, the cell is considered as water equivalent, therefore the value of  $\rho$  is 1) and  $F = \text{the particle fluence}$ .

### Preparation, staining and scoring technique of metaphase chromosomes

Chromosomes were arrested at metaphase with 2 h Colcemid treatment (0.1 μg/mL). The cells were then trypsinized, washed twice with PBS, treated hypotonic solution (0.56% KCl, 20 min at 37 °C) and fixed in methanol:acetic acid (3:1) fixative. Finally, cell suspension was dropped onto a chilled, pre-cleaned glass microscope slides. The slides were stained in 4% Giemsa in PBS (pH 7.0). 100 metaphase spreads were scored under 1000x magnification. Metaphase spreads were analyzed for chromosome-type aberrations— including chromosome-type breaks, double-minutes, dicentric, and rings and chromatid-type aberrations including chromatid-type breaks and chromatid-type exchanges. Special considerations were made for ring and dicentric chromosomes. For instance, when a dicentric or ring chromosome was scored, one acentric fragment was subtracted from the number of fragments found in the cell.

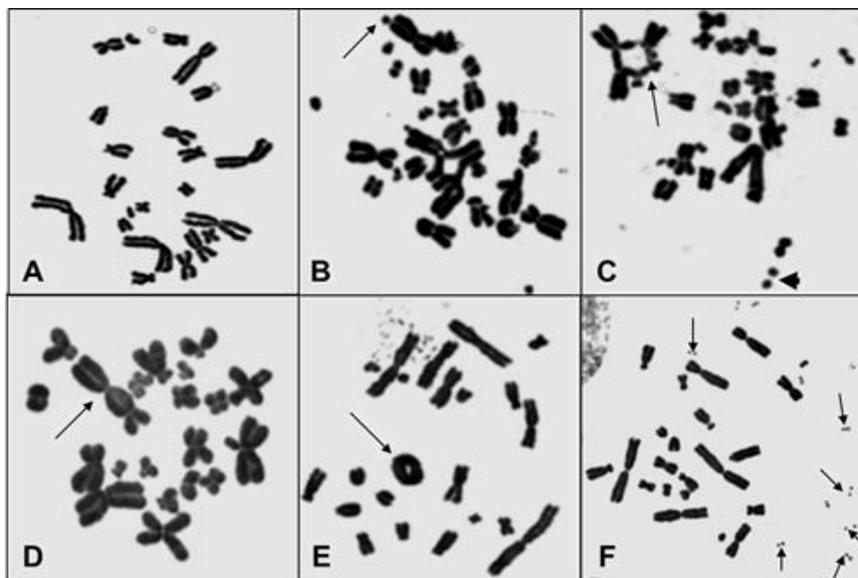
### Statistical Analysis

Except for 48 h after <sup>16</sup>O-irradiation, all the data of CAs were fitted with a linear equation ( $Y = \alpha D$ ). Standard errors on the

frequencies were calculated by  $\sqrt{a/A}$ , where 'a' is the number under consideration and 'A' is the total number of cells analyzed [12].

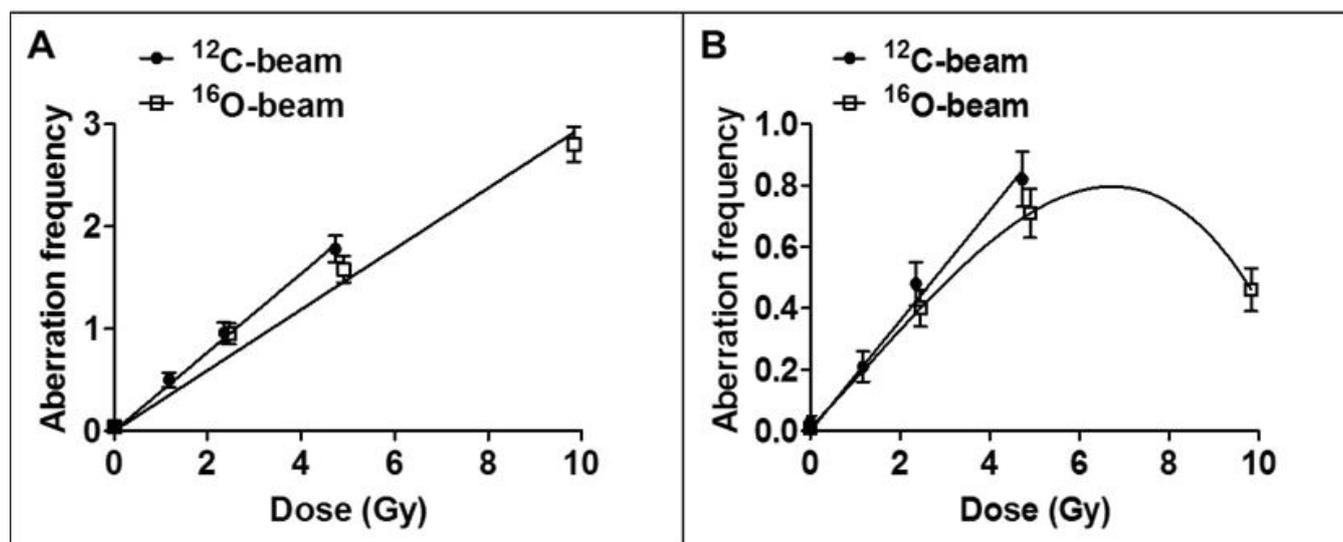
## RESULTS

### Dose-Response Relationship for Chromosomal Aberrations



**Figure 1.** Representative photomicrographs of metaphase spread of M5 cells. Panel A: showing normal metaphase spread with 22 chromosomes. Panel B: showing chromatid-type break indicated by arrow. Panel C: representing chromatid-type exchange (indicated by arrow) and chromosome-type break (indicated by arrow head). Panel D: showing dicentric chromosome indicated by arrow. Panel E: showing ring chromosome (indicated by arrow). Panel F: showing double-minute chromosomes (indicated by arrows).

**Figure 1** showing representative photomicrographs of different types of structural aberrations. Spectrum of different structural aberrations is shown in **Table 1**. We observed a dose-dependent increase in various types of structural CAs after exposure to both  $^{12}\text{C}$ - and  $^{16}\text{O}$ -beams.



**Figure 2.** Dose-response curve for chromosomal aberrations in M5 cells. Panel A and B showing frequency of total chromosomal aberrations in M5 cells exposed to  $^{12}\text{C}$ - and  $^{16}\text{O}$ -beam at 24 h and 48 h, respectively.

**Figure 2** showing the dose-response curves for total CAs at 24 h and 48 h. At 24 h, a linear correlation between total CAs frequency and radiation dose was observed for both radiation types, however, the frequency of total CAs was higher in  $^{12}\text{C}$ -irradiated cells. A similar dose-dependent linear increase in total CAs was observed in  $^{12}\text{C}$ -irradiated M5 cells at 48 h. However, at 48 h after  $^{16}\text{O}$ -irradiation, frequency of total CAs did not follow linear increase; rather a sharp decline in total aberration frequency was

observed after exposure to 9.83 Gy (**Figure 1**). At 48 h, the dose-response curve for total CAs revealed that <sup>12</sup>C-beam induces more chromosomal damage than the same dose of <sup>16</sup>O-beam (**Figure 1 and Table 1**).

	Tfx	Fluence (particle/cm <sup>2</sup> )	Dose (Gy)	Chromatid-type aberrations		Chromosome-type aberration				Total	
				Ctb	Cte	Csb	Dic	Dimn	Ring		
<sup>12</sup> C-beam	24 h	0	0	0.00 ± 0.00	0.03 ± 0.02	0.03 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.06 ± 0.02	
				0	3	3	0	0	0	6	
		1 × 10 <sup>6</sup>	1.18	0.02 ± 0.01	0.09 ± 0.03	0.18 ± 0.04	0.10 ± 0.03	0.11 ± 0.03	0.00 ± 0.00	0.50 ± 0.07	
				2	9	18	10	11	0	50	
		5 × 10 <sup>6</sup>	2.36	0.07 ± 0.03	0.16 ± 0.04	0.35 ± 0.06	0.17 ± 0.04	0.19 ± 0.04	0.02 ± 0.01	0.96 ± 0.10	
				7	16	35	17	19	2	96	
		1 × 10 <sup>7</sup>	4.73	0.15 ± 0.04	0.21 ± 0.05	0.52 ± 0.07	0.23 ± 0.05	0.62 ± 0.08	0.05 ± 0.02	1.78 ± 0.13	
			15	21	52	23	62	5	178		
	48 h	0	0	0.02 ± 0.01-2	0.00 ± 0.00	0.01 ± 0.01-1	0.00 ± 0.000	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.02	
					0			0	0	3	
		1 × 10 <sup>6</sup>	1.18	0.02 ± 0.01-2	0.04 ± 0.02	0.04 ± 0.02-4	0.05 ± 0.02-5	0.05 ± 0.02	0.01 ± 0.01	0.21 ± 0.05	
					4			5	1	21	
		5 × 10 <sup>6</sup>	2.36	0.08 ± 0.03-8	0.08 ± 0.03-8	0.02 ± 0.01-2	0.08 ± 0.03	0.20 ± 0.04	0.02 ± 0.01	0.48 ± 0.07	
					8	8	2	8	20	2	48
<sup>16</sup> O-beam	24 h	0	0	0.01 ± 0.01	0.00 ± 0.00	0.03 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.02	
					1	0	3	0	0	4	
		1 × 10 <sup>6</sup>	2.46	0.05 ± 0.02	0.18 ± 0.04	0.23 ± 0.05	0.21 ± 0.05	0.26 ± 0.05	0.02 ± 0.01	0.95 ± 0.10	
					5	18	23	21	26	2	95
		5 × 10 <sup>6</sup>	4.91	0.12 ± 0.03	0.31 ± 0.06	0.33 ± 0.06	0.40 ± 0.06	0.38 ± 0.06	0.04 ± 0.02	1.58 ± 0.13	
					12	31	33	40	38	4	158
		1 × 10 <sup>7</sup>	9.83	0.17 ± 0.04	0.60 ± 0.08	0.46 ± 0.07	0.77 ± 0.09	0.78 ± 0.09	0.02 ± 0.01	2.80 ± 0.17	
				17	60	46	77	78	2	280	
	48 h	0	0	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	
					0	1	0	0	0	1	
		1 × 10 <sup>6</sup>	2.46	0.07 ± 0.03	0.11 ± 0.03	0.04 ± 0.02	0.04 ± 0.02	0.13 ± 0.04	0.01 ± 0.01	0.40 ± 0.06	
					7	11	4	4	13	1	40
		5 × 10 <sup>6</sup>	4.91	0.09 ± 0.03	0.17 ± 0.04	0.05 ± 0.02	0.11 ± 0.03	0.27 ± 0.05	0.02 ± 0.01	0.71 ± 0.08	
					9	17	5	11	27	2	71
1 × 10 <sup>7</sup>		9.83	0.06 ± 0.02	0.07 ± 0.03	0.03 ± 0.02	0.05 ± 0.02	0.24 ± 0.05	0.01 ± 0.01	0.46 ± 0.07		
			6	7	3	5	24	1	46		

### Time Kinetics of CAs

Aberration frequencies substantially decrease with the incubation time for both radiation types as evident from **Table 1**. For example, we observed approximately 54% and 84% decrease in total CAs at 48 h as compared to 24 h in M5 cells exposed to 4.73 Gy of  $^{12}\text{C}$ -beam and 9.83 Gy of  $^{16}\text{O}$ -beam, respectively (**Table 1**).

### Aberration Spectrum

Analysis of aberration spectrum at various post-irradiation times revealed that the frequency of chromosome-type aberrations were considerably higher than chromatid-type aberration for both radiation types (**Table 1**). For example, the frequency of total chromosome-type aberration at 24 h was about 3.9 and 2.6 times higher than the chromatid-type aberration after exposure to 4.73 Gy of  $^{12}\text{C}$ -beam and 9.83 Gy of  $^{16}\text{O}$ -beam, respectively. Interestingly, the frequency of chromosome-type breaks was higher than dicentrics at 24 h in  $^{12}\text{C}$ -irradiated cells, while more dicentrics were observed than chromosome-type breaks in cells exposed to  $^{16}\text{O}$ -beam (**Table 1**).

## DISCUSSION

Quantification of CAs is an important parameter to better understand the radiobiological effects of high-LET radiation. High-LET radiation-induced complex DSBs has a direct bearing on cellular repair and proliferation kinetics<sup>[9]</sup>. We demonstrated that high-LET 7Li-,  $^{12}\text{C}$ -, and  $^{16}\text{O}$ -beam can kill chemo-radio-resistant M5 cells more effectively than 60Co gamma-rays and that cell killing is strongly correlated with the frequency of CAs<sup>[6-8]</sup>. Therefore, a comparative study of CAs after exposure to various high-LET radiations will help to understand how the physical qualities of radiation influence the structural abnormality of chromosomes. This knowledge will be crucial to strategize treatment planning.

In general, the current study demonstrated that  $^{12}\text{C}$ -beam causes linear dose-dependent increase in total CAs at 24 h and 48 h. However, after  $^{16}\text{O}$ -irradiation, linear dose-dependent increase was only observed at 24 h, not at 48 h. At 48 h, the frequency of total CAs increased linearly up to 4.91 Gy, then steeply declined at 9.83 Gy after  $^{16}\text{O}$ -irradiation. This decline may be due to the elimination of heavily damaged cells from the population. We observed similar decline at 48 when V79 cells were exposed to 9.83 Gy of  $^{16}\text{O}$ -beam<sup>[8]</sup>. The slope values of dose-response curves,  $0.38 \pm 0.01$  and  $0.29 \pm 0.01$  for  $^{12}\text{C}$ - and  $^{16}\text{O}$ -beam at 24 h, respectively, clearly indicate  $^{12}\text{C}$ -beam induces more chromosomal damage than  $^{16}\text{O}$ -beam. These data suggests that particle radiation with relatively lower LET value may induce more chromosomal damage. This validates previous findings of Tenhumberg et al.<sup>[13]</sup>, who noticed  $^{12}\text{C}$ -ions with a relatively lower LET value ( $153.3 \text{ keV}/\mu\text{m}$ ) was more effective than Ni-ions ( $2455 \text{ keV}/\mu\text{m}$ ) in inducing cytogenetic damage in human fibroblast cells<sup>[13]</sup>. Thus, it is evident that a strong correlation exists between LET values and aberration yield.

We also noticed a substantial decline in total CAs in M5 cells with the increase in post-irradiation incubation time, which corroborates our previous observations with V79 cells<sup>[8]</sup>. However, the rate of decline varies depending on the aberration types. For example, the decline in chromatid-type breaks was considerably less than the chromosome-type breaks. Approximately 46% and 94% decrease in chromatid- and chromosome-type breaks, respectively, was observed at 48 h after exposure to 4.73 Gy of  $^{12}\text{C}$ -beam. Similar trend was also noticed after 9.83 Gy of  $^{16}\text{O}$ -irradiation. Interestingly, at 24 h,  $^{12}\text{C}$ -beam induced more chromosome-type breaks than dicentrics, while  $^{16}\text{O}$ -irradiation showed complete opposite trend. The frequency of chromatid-type exchange was substantially higher than chromatid-type breaks for both radiation types. The ratio of chromatid-type exchange to chromatid break at 24 h was considerably higher after  $^{16}\text{O}$ - than  $^{12}\text{C}$ -irradiation. These data suggests that radiation quality strongly influence aberration spectrum.

Both  $^{12}\text{C}$ - and  $^{16}\text{O}$ -beams induce more chromosome- than chromatid-type aberrations, which suggest that the confluent M5 cells were in G1 phase during irradiation. This could be due to contact inhibition of cell proliferation. Irradiation of cells in G0/G1 phase is also known to favor chromosome-type aberrations<sup>[14]</sup>. Interestingly, we observed chromosome- and chromatid-type aberrations in the same metaphase spread, which is contrary to the general belief and suggests that these chromatid-type aberrations are induced in pre-DNA synthetic stage. This could be the result of alkali-labile sites or single strand DNA breaks induced in G1 cells after high-LET radiation; since substantial number of single strand breaks remain as "unrepaired lesions" and lead to formation of chromatid-type aberrations. Ritter et al. also reported presence of both chromosome- and chromatid-type aberrations in the same metaphase spread of V79 cells exposed to high-LET Ar-ions ( $\text{LET} = 1840 \text{ keV}/\mu\text{m}$ ) in G1 phase<sup>[15]</sup>. Presence of both chromosome- and chromatid-type aberration after high-LET radiation, unlike low-LET gamma or X-rays, is expected to increase the overall aberration burden, leading to cell-cycle perturbations and mitotic delay, and ultimately enhanced mitotic death.

## CONCLUSION

Resistance to chemotherapeutic drugs and therapeutic radiation are the major limiting factors for successful cancer treatment. Methotrexate is still employed to treat certain types of human malignancies and radiation is used for treating half of the cancer patients, which may make the tumor cells eventually become resistant to therapy. Using a methotrexate plus radiation resistant cell line (M5), current study clearly demonstrated that quality of high-LET radiation determine the extent and spectrum of chromosomal damage in a time-dependent manner and  $^{12}\text{C}$ -beam is more effective in inducing CAs than  $^{16}\text{O}$ -beam. These findings

will certainly help to design the strategy to treat cancer patient with high-LET radiation.

### ACKNOWLEDGEMENTS

This work was supported by Seed Fund from College of Pharmacy, University of Arkansas for Medical Sciences [RP]; UGC Funded University Project (New Delhi, India) [RP]; and National Institutes of Health (P20 GM109005) [MH-J]. These agencies had no role in the study design, data analysis, or the preparation of this manuscript. The senior author (RP) is thankful to Prof. N.P. Bhattacharyya, C&MB Division, Saha Institute of Nuclear Physics, Salt Lake, Kolkata 700064, for kindly providing the M5 cell strains and also to all the members of Pelletron group at Inter University Accelerator Center (New Delhi, India) for their sincere help during high-LET irradiation.

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