Journal of Innovations in Pharmaceutical and Biological Sciences (JIPBS)
ISSN: 2349-2759
Available online at www.jipbs.com

Research article

A12B4C3, an inhibitor of polynucleotide kinase/phosphatase enhances radio-sensitivity in PC-3 cells exposed to carbon ion beam

Pallavi Srivastava[1], Asitikantha Sarma[2], Chandra Mohini Chaturvedi[1*]

[1]*Department of Zoology, Institute of Science, Banaras Hindu University, Varanasi, Uttar Pradesh, India*
[2]*Radiation Biology Laboratory, Inter University Accelerator Centre, New Delhi, India.*

Key words: High LET radiation, PNKP, PC-3, Gamma radiation.

***Corresponding Author: Chandra Mohini Chaturvedi,** Department of Zoology, Institute of Science, Banaras Hindu University, Uttar-Pradesh, Varanasi – 221005, India .

Abstract

Cancer recurrence is a major problem of current radiotherapies like gamma radiation or High LET (Linear Energy Transfer) radiation. This problem can be overcome by the use of radio-modulator which will enhance cell killing and/or prevent cell regeneration after the treatment. Polynucleotide kinase / phosphatase (PNKP) plays an important role in DNA repair pathway. Accordingly, its inhibition can hamper the DNA damage repair processes and ultimately can suppress the recurrence of cancer. In this context, we have reported that following 24 hr ^{12}Carbon ion beam combined with PNKPi (PNKP inhibitor) as radio-modulator enhanced the radio-sensitivity. Present study will elucidate the effect after 48 hr of ^{12}Carbon ion beam in combination with PNKPi. We have checked the PC-3 cell viability through MTT assay, cell death mode by DAPI staining and cell cycle analysis through flow cytometry after 48 hr of irradiation (^{12}Carbon ion beam having energy 62 MeV; equivalent to 5.16 MeV/nucleon with entrance LET 287 kev/μm) and in combination with PNKPi as radiomodulator. It was observed that PC-3 cell viability decreased following apoptosis when carbon ion beam was combined with PNKPi. Further, the cell cycle arrest was enhanced at S phase in combined treatment group compared to only irradiated cells. These findings suggest that, after 48 hr of carbon ion irradiation PNKP inhibition could enhance cellular radiosensitivity in prostate cancer cell line PC-3 by suppressing DNA damage repair pathway. The synergistic effect of PNKPi and carbon ion irradiation may be a promising method to avoid recurrence of cancerous cells following High LET carbon-ion radiation therapy.

Introduction

World wide prostate cancer prevalence is highest among American men of Caucasian and African origin [1]. In USA, prostate cancer incidence is 19% and it is the second most common cause of death due to cancer in men after lung and bronchus cancer. Prostate cancer (PCa) alone accounting for almost one in five new diagnoses [2]. Cancer is rising as a major killer in developing countries like India[3].Prostate is the second most leading site of cancer among males, in big Indian cities like Delhi, Kolkata, Pune and Thiruvananthapuram and third leading site of cancer in Bangalore and Mumbai. It is among the top ten leading sites of cancers in the rest of the population based cancer registries (PBCRs) of India. PCa incidence rate are constantly and rapidly increasing in all the PBRCs. The cancer projection data reveals that the number of cases will become doubled by 2020 [4]. Major obstacle in the therapy of cancer is recurrence after radiotherapy and it is due to inherent damage repair property of the cell. This provides a strong rationale for exploration of potential inhibitors which can suppress the DNA damage repair pathway of cancerous cells and thus can prevent cell regrowth. PNKP (polynucleotide kinase/phosphatase) plays vital roles in mammalian base excision repair (BER), single strand break (SSB) repair, double strand break (DSB) repair and non-homologous end joining (NHEJ) making it an attractive therapeutic target [5].

In recent years, level of interest has increased to inhibit DNA-repair pathways as an approach to potentiate the radio-therapeutic cancer treatments. Inhibitors of several enzymes involved in the repair of DNA strand breaks works as radiomodulator and are currently at various stages of the drug development process [6-7]. Therefore, targeting signaling networks involved in DNA break repair is a promising approach for enhancing cellular radiosensitivity.

High LET (Linear Energy Transfer) ^{12}Carbon ion radiation has many potential advantages over the low LET radiation such as gamma radiation or X-rays. Heavy ion beam or ^{12}Carbon ion radiation as typical high LET radiation creates cluster damage to DNA and produces high Relative Biological Effectiveness (RBE), utilizing Spread Out Bragg Peak (SOBP) and resulting in an increased therapeutic ratio [8]. A major reason for the physical selectivity of the inverted dose profile is a sharp longitudinal dose drop at the end of the particle range,

and lateral scattering can be neglected for heavy particles such as carbon ions. This permits dose escalation within the tumor which might result in a better tumor control [9]. Radiation induces DNA breaks with incompatible termini i.e. 3′-phosphate and 5′-hydroxyl moieties which must be processed for SSBs & DSBs repair pathway to complete repair. These lesions create a barrier for DNA polymerase and ligase to replace missing bases and seal the breaks because these enzymes have a strict requirement for the presence of a 3′-OH & in addition DNA ligases requires a 5′-PO_4 group. It is now clear that the major enzyme responsible for phosphorylation of 5′-OH termini and dephosphorylation of 3′-PO_4 termini in human cells is polynucleotide kinase/phosphatase (hPNKP) [10]. hPNKP or PNKP contains both 5′kinase and 3′-phosphatase activities required for restoration of 3′-hydroxyls and 5′-phosphates needed to seal the broken DNA.

Till now most of the focus for radiomodulator has centered on the inhibitors of poly [ADP-ribose] polymerase (PARP) and protein kinase (PK). It has been suggested that the terminal base pairs of double-stranded substrates near the 3′-phosphate are destabilized by PNKP to allow substrate access to the active site [5]. It has also been suggested that inhibiting the PNKP may sensitize cancer cells to chemotherapy and radiotherapy [11-12]. Allinson et al suggested PNKP as a potential target in the treatment of cancer [12]. Inhibition of PNKP represents a DNA repair targeted therapy, which sensitizes cancer cells to DNA damage and enhances anticancer potential. Obviously, new inhibitory compounds need to be identified and optimized for clinical use.

The hPNKP inhibitor, A12B4C3 is reported to inhibit the mammalian PNKP activity. It disrupts the secondary structure of PNKP and acts as a noncompetitive inhibitor that allosterically regulates the phosphatase activity of human PNKP [13]. A12B4C3 is 2-(1-hydroxyundecyl)-1-(4-nitrophenylamino)-6-phenyl-6,7a-dihydro-1Hpyrrolo [3,4-b]pyridine-5,7(2H,4aH)-dione, a polysubstituted piperidine (Figure1) [14-15]. A12B4C3 has ability to inhibit phosphatase activity of PNKP *in vitro* and it was evidenced that it radiosensitizes the wild type A-549 cells to ionizing radiation but not A-549 PNKP knockdown cells suggesting PNKP is its only target. It enhanced the radiosensitivity in human A549 lung carcinoma and MDA-MB-231 breast adenocarcinoma cells at a very low non-toxic dose 1 μmol/L [14]. Therefore we have selected A12B4C3 to inhibit the PNKP activity. A12B4C3 treatment was shown to radio-sensitizes human A549 cells, MDA-MB-231cells and human myeloid leukemia cells with γ-radiation but has not been studied in a radio resistant cells with C-ion radiation [16-17].

In our previous study we have reported that after 24 hr of ^{12}C ion exposure, A12B4C3 (an inhibitor of PNKP activity) significantly inhibited the cell survival in PC-3 prostate cancer cells [18]. This data intrigue us to check the effect of A12B4C3 after 48 hr of carbon ion exposure to compare and assures the post radiation effect in PC-3 cells. Hence, present study was designed to monitor the regrowth/recurrence in PC-3 cells after 48 hr of carbon ion radiation in combination with PNKPi (PNKP inhibitor) i.e. A12B4C3 as radiomodulator.

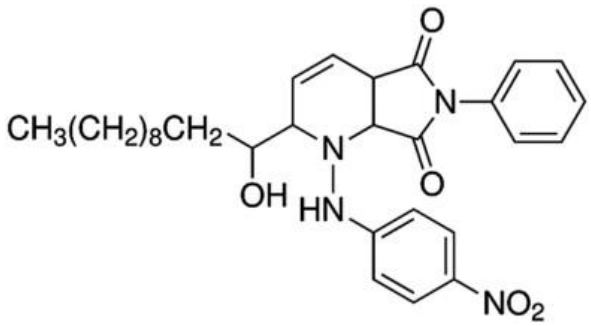

Figure 1. Chemical structure of A12B4C3 [2-(1-hydroxyundecyl)-1-(4-nitrophenylamino)-6-phenyl-6,7a-dihydro-1H-pyrrolo[3,4-b]pyridine-5,7(2H,4aH)-dione, 4a,7a-Dihydro-2-(1hydroxyundecyl)-1-[(4-nitrophenyl) amino]-6-phenyl-1H-pyrrolo[3,4-b]pyridine-5,7(2H,6H)-dione].

Experimental

Materials and methods

Cell culture

Prostate cancer PC-3 cells were purchased from our National repository NCCS (National Centre for Cell Science, Pune, India) and maintained in nutrient mixture F-12 Ham, K medium (Himedia, India) supplemented with 10% fetal bovine serum (Himedia, India) in presence of antibiotic solution (Himedia, India) at 5% CO_2 in a humidified atmosphere at 37°C. 70-80% confluent cultured cells were used for experiment.

Treatment

0.5×10^5 cells/plate were treated with the PNKP inhibitor A12B4C3 (Sigma Aldrich, USA) 2-4 hr. before irradiation. A12B4C3 (PNKPi) stock solution was made in dimethyl sulfoxide (DMSO) and dilution was prepared in culture medium. We have used very low concentration of PNKPi 10μM based on our previous study [18].

Carbon ion beam/radiation

15 UD Pelletron accelerator at Inter University Acclerator Centre (IUAC), New Delhi, India, was utilized for irradiation of cells. Cells were cultured and maintained in Radiation Biology Laboratory at IUAC, New Delhi. The irradiation of cells was done using heavy ion irradiation facility ASPIRE software, where the dosimetry is done using silicon surface barrier detectors [19-20]. ^{12}C beam with 85MeV (equivalent to 7.08 MeV/nucleon) energy from the accelerator was used. The energy of the beam on

the cell surface was 62 MeV (equivalent 5.16 MeV/nucleon) with entrance LET 287 kev/μm as calculated by SRIM software [20-21]. The beam flux was maintained at about 2 x 10^5 particles/sq.cm/sec. 2Gy and 4Gy radiation dose was used for the present study. The dose in Gy was calculated using the following standard relation, where the cell is taken to be water equivalent.

Dose (Gy) = 1.6 x 10^{-9} x LET (keV/um) x Fluence (particles/sq. cm) [22].

MTT assay

Cell viability was evaluated by MTT assay according to Plumb *et al.* (1989) [23]. MTT tetrazolium salt was dissolved in PBS (pH 7.4) at 5 mg/ml. After 48hr incubation period, 30 μl MTT solution was added to each well, mixed thoroughly and all plates were again placed in the incubator. After 4 hr incubation, 100 μl DMSO was added to each well and mixed thoroughly in order to dissolve the formed blue crystals of formazan. All plates were placed on shaker for 5 min. The absorbance was measured at a wavelength of 490 nm using VICTOR™*X*5 multilabel Plate Reader (PerkinElmer).

Viability of cells was calculated by using the following formula:

% Viability = OD of PNKPi treated cells + OD of radiation treated cells /OD of control cells (without PNKPi) X 100.

Apoptotic body formation

Apoptotic body formation was observed under flurorescence microscope after staining with Prolong^R Gold Antifade reagent. % of apoptotic bodies were counted (approx. 150 cells at each dose) after 48 hr of C-ion irradiation.

Cell cycle distribution by FACS

Propidium Iodide (PI) staining was used to analyze the phases of cell cycle. After irradiation, cells were incubated for 48hr and then collected in medium by scraping, rinsed with PBS, centrifuged, put in 70 % precooled ethanol, fixed at 4^0C overnight. After fixation, cells were washed thrice with cold PBS and then stained with staining solution (10μg/ml Propidium Iodide, 100μg/ml of DNase-free RNase and 0.1% v/v triton-100) and incubated at 37^0C for 15 min. The phases of cell cycle were detected on the BD Accuri™ Flow Cytometer (B.D. Biosciences, USA) and data analyzed by BD Accuri C6 software.

Statistical analysis

Data were averaged from at least three biological repeats and presented as mean ± standard deviation wherever required. Different samples means were compared by one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple comparison tests. P<0.05 was considered significant. All statistical analysis was done using Prism ver. 5 (GraphPad Software Inc., USA).

Results and discussion

Effects on cell morphology and viability when carbon ion radiation was combined with PNKPi

Our previous study suggested that at 24 hr time point, PNKPi enhanced the radiosensitivity in PC-3 cells after exposure of carbon ion radiation [18]. Present study reveals that after 48 hr of carbon ion radiation appearance of cells changed when combined with PNKPi (Figure 2A). Cell viability data showed that viability of cells decreased to 71.32±0.02%, 61.48±0.12%, 47.38±0.04% and 35.36±0.02% in 2Gy, 2Gy+PNKPi, 4Gy and 4Gy+PNKPi group as compared to control (100%) and 62.77±0.02%, 75.14±0.03% in the 2Gy+PNKPi and 4Gy+PNKPi groups as compared to cells that were irradiated only i.e. 2Gy and 4Gy respectively (Figure 2B).

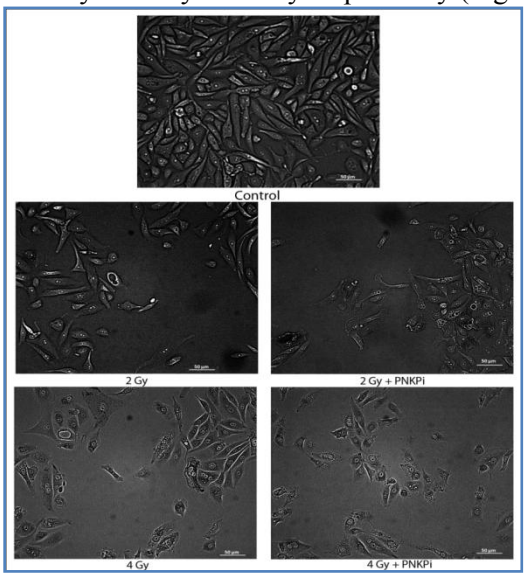

Figure 2 (A)

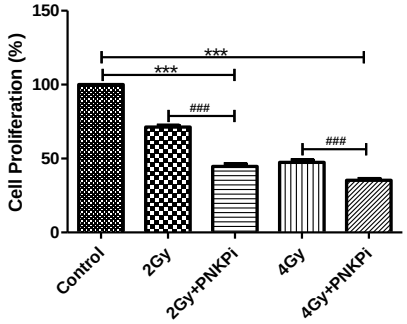

Figure 2 (B)

Figure 2. (A) Morphological changes of PC-3 cells with and without PNKPi (A12B4C3) treatment after 48 hr of 2Gy and 4 Gy ^{12}C beam (B)Cell viability (%) decreased after 48 hr of radiation when ^{12}C beam is combined with PNKPi. ***p<0.001, significance of difference from control and ###p<0.001, significance of difference from only irradiated group respectively.

Combined treatment of carbon ion radiation and PNKPi stimulate cell death through apoptotic body formation

Many reports suggested that carbon ion radiation induced cell death through apoptosis and independent of p53 status [24]. Since PC-3 cell line is null for p53 so, it is undergoing p53-independent apoptosis. After 48 hr of irradiation apoptotic body formation was significantly higher (2.61±1.02, 1.72±0.03 fold higher) in cells which were treated with PNKPi in combination with C-ion irradiation as compare to only irridiated cells i.e. 2Gy and 4Gy. We found 2.83±0.72, 6.00±0.57, 9.5±0.98, 16.55±2.02 fold increase in number of apoptotic bodies in 2Gy, 2Gy+PNKPi, 4Gy and 4Gy+PNKPi groups as compared to control (Figure 3).

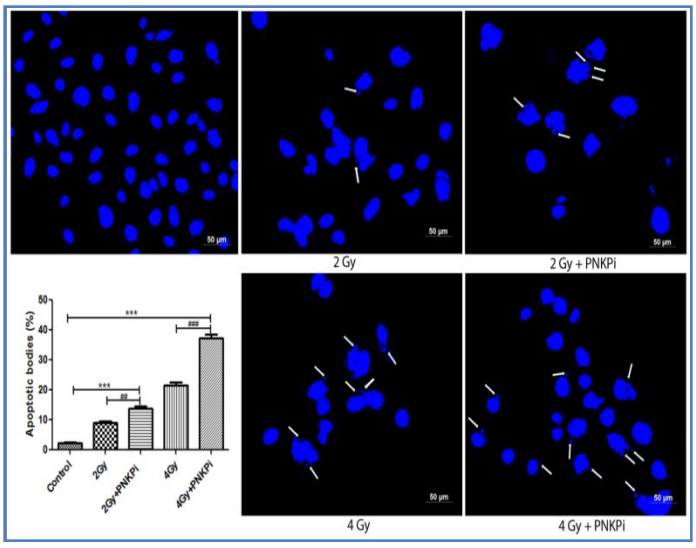

Figure 3. Arrow showing apoptotic body formation after 48 hr of ^{12}C beam in the presence and absence of PNKPi. Bar diagram illustrating pattern of apoptotic body(%) after 48 hr of radiation.*p<0.001, significance of difference from control and ##p<0.01, ###p<0.001, significance of difference compare to the only irradiated group.**

Carbon ion radiation induces significant S phase arrest after 48 hr when combined with PNKPi

Cell cycle progression is controlled by many checkpoints at different phases; in which G1/S check point is one of the most critical for control of cell proliferation. Failure of this check point can lead to abnormal growth or apoptosis. Prostate cancer cells (PC-3) showed, a significant cell cycle arrest (1.43±0.02, 1.23±0.01 fold change) at S phase after 48 hr of carbon ion radiation in the presence of PNKPi as compare to only irradiated cells i.e. 2Gy and 4 Gy. We observed 1.21±0.04, 1.73±0.08, 2.13±0.13, 2.62±0.13 fold enhancement at S phase arrest in 2 Gy, 2Gy+PNKPi, 4Gy, 4Gy+PNKPi groups as compared to control cells (Figure 4).

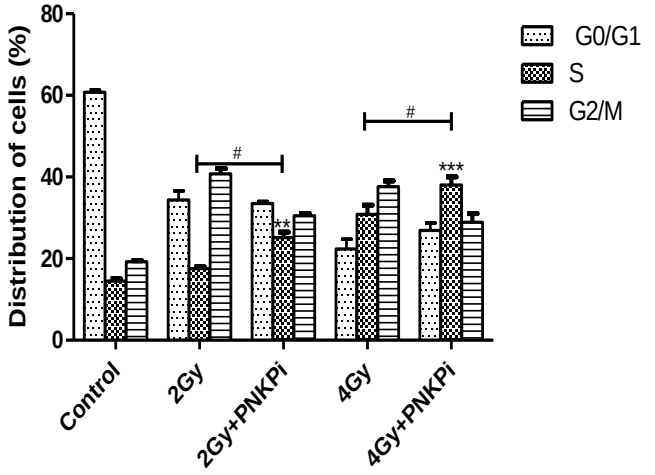

Figure 4. FACS analysis showing distribution of cells % after 48 hr of radiation (cell cycle arrest at different phases). S phase arrest was enhanced in PNKPi treated group comapred to control cells. **p<0.01; ***p<0.001, significance of difference compared to control and #p<0.05 in comparison with the only irradiated group.

Conclusion

In view of the fact that recurrence is major obstacle in the treatment of cancer, targeting DNA repair process could be an attractive approach for cancer therapy. Present experimental study confirmed that, PNKP inhibition by A12B4C3 may be a suitable approach to reduce the recurrence after exposure of carbon ion radiation in prostate cancer cell PC-3. It is also suggested that 10μM, a low concentration of A12B4C3 is effective after 48 hr of carbon ion radiationin 2 Gy-4Gy range. However, further understanding of the molecular mechanism behind the radio-sensitization of PNKPi will strengthens the use of PNKP inhibitor A12B4C3 as a possible radio-modulator to reduce the recurrence of cancerous cells following radiotherapy.

Acknowledgment

We are highly thankful to Inter University Accelerator Centre, New Delhi for financial support grant UFR-56310 and National Institute of Immunology, New Delhi for providing their FACS facility. Council of Scientific & Industrial Research (CSIR), India, is highly acknowledged for senior research fellowship to Pallavi Srivastava.

References

1. Haas GP, Delongchamps N, Brawley OW, Wang CY and Roza GDL: The worldwide epidemiology of prostate cancer: perspective from autopsy studies. Cancer Journal of Urology 2008; 15:3866-3871.
2. Siegel R, Miller K and Jemal A: Cancer statistics. CA Cancer J Clin 2018; 68:7-30.
3. Shukla P, Mathur V, Kumar A, Khedgikar V, Teja BV, Chaudhary D, KushwahaP, Bora HK, Konwar R, Trivedi R and Mishra PR: Nanoemulsion Based Concomitant Delivery of Curcumin and Etoposide: Impact on Cross Talk Between Prostate Cancer Cells and Osteoblast

During Metastasis. Journal of Biomedical Nanotechnology 2014; 10: 3381–3391.
4. Jain S, Saxena S and Kumar A: Epidemiology of prostate cancer in India. Meta gene 2014; 2: 596–605.
5. Coquelle N, Shahriari ZH, Bernstein N, Green R and Glover JNM: Structural basis for the phosphatase activity of polynucleotide kinase/phosphatase on single and double-stranded DNA substrates. PNAS 2011; 108:21022–21027.
6. Davidson D, Amrein L, Panasci L and Aloyz R: Small molecules, inhibitors of DNA-PK, targeting DNA repair, and beyond. Frontiers in pharmacology 2013; 4:1-7.
7. Curtin NJ and Szabo C: Therapeutic applications of PARP inhibitors: anticancer therapy and beyond. Mol Aspects Med 2013; 34:1217-56.
8. Fukumura A, Tsujii H, Kamada T*et al*: Carbon-ion radiotherapy: clinical aspects and related dosimetry. Radiat. Prot. Dosimetry 2009; 137:149–55.
9. Kramer M, Scholz M: Rapid calculation of biological effects in ion radiotherapy. Phys Med Biol 2006; 5:1959-1970.
10. Schellenberg MJ and Williams RS: DNA end processing by polynucleotide kinase/phosphatase. PNAS 2011; 108:20855–20856.
11. Mereniuk TR, EI Gendy MA, Mendes-Pereira AM, Ana MMP, Lord C J, Ghosh S, Foley E, Ashworth A and Weinfeld M: Synthetic lethal targeting of PTEN-deficient cancer cells using selective disruption of polynucleotide kinase/phosphatase. Mol Cancer Ther 2013; 12:2135–2144.
12. Allinson SL: DNA end-processing enzyme polynucleotide kinase as a potential target in the treatment of Cancer. Future Oncology 2010; 6:1031-42.
13. Freschauf GK, Mani RS, Mereniuk TR, Fanta M, Virgen CA, Dianov GL et al: Mechanism of action of an imidopiperidine inhibitor of human polynucleotide kinase/phosphatase. J. Biol. Chem 2010; 285: 2351–2360.
14. Freschauf GK, Karimi-Busheri F, Ulaczyk-Lesanko A, Mereniuk TR, Ahrens A, Koshy JM, Rasouli-Nia A, Pasarj P, Holmes CFB, Rininsland FR, Hall DG and Weinfeld M: Identification of a small molecule inhibitor of the human DNA repair enzyme polynucleotide Kinase/Phosphatase. Cancer Res 2009; 69: 7739-7746.
15. Zereshkian A, Leyton JV, Cai Z, Bergstrom D, Weinfeld M and Reilly RM: The human polynucleotide kinase/phosphatase (hPNKP) inhibitor A12B4C3 radiosensitizes human myeloid leukemia cells to Auger electron-emitting anti-CD123 111In-NLS-7G3 radioimmuno conjugates. Nucl Med Biol 2014; 41: 377–383.
16. Lee Y, Sunada S, Hirakawa H, Fujimori A, Nickoloff JA and Okayasu R: TAS-116, a novel hsp90 inhibitor, selectively enhances radiosensitivity of human cancer cells to x-rays and carbon ion radiation. Mol Cancer Ther. 2017; 16: 16-24.
17. Kelley MR, Logsdon D and Fishel ML: Targeting DNA repair pathways for cancer treatment: what's new?. Future Oncology 2015; 10:1215-1237.
18. Srivastava P, Sarma A and Chaturvedi CM: Targeting DNA repair with PNKP inhibition sensitizes radioresistant prostate cancer cells to high LET radiation. Plos One 2018; 13:1-14.
19. Ghorai A, Sarma A, Chowdhury P and Ghosh U: PARP-1 depletion in combination with carbon ion exposure significantly reduces MMPs activity and overall increases TIMPs expression in cultured HeLa cells. Radiation Oncology 2016; 11: 1-13.
20. Kothari A, Barua P, Archunan M, Rani K, Subramanian ET, Pujari G *et al*: ASPIRE: An automated sample positioning and irradiation system for radiation biology experiments at Inter University Accelerator Centre, New Delhi. Radiation Measurements 2015; 76:17-22.
21. Jiegler JF, Bierserk JP and Littmark U: Stopping and ranges of ions in matter. Pergamon Press, New York.
22. Kraft-Weyrather W, Kraft G, Ritter S, Scholz M and Stanton JA: The preparation of biological targets for heavy-ion experiments up to 20MeV/u: Nuclear Instruments and Methods in Physics Research A 1989; 282:22- 27.
23. Plumb JA, Milroy R and Kaye S B: Effects of the pH dependence of 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl-tetrazolium bromide–formazan absorption on chemosensitivity determined by a novel tetrazolium- based assay. Cancer Research 1989; 49: 4435–4440.
24. Mori E, Takahashi A, Yamakawa N, Kirita T and Ohnishi T: High LET heavy ion radiation induces p53 independent apoptosis. J Radiat Res 2009; 50:37–42.