

CARBON IONS INDUCE DNA DOUBLE STRAND BREAKS AND APOPTOSIS IN HTB140 MELANOMA CELLS

by

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This study was conducted in order to evaluate the ability of carbon ions to induce DNA double-strand breaks and apoptosis in the radio-resistant human HTB140 melanoma cells. The cells were irradiated with ¹²C ions having the linear energy transfer of 258 keV/ m. Irradiations were performed in the dose range from 2 to 16 Gy. Induction of DNA double-strand breaks was evaluated 2 hour after irradiation through expression of γ H2AX protein. Increased level of γ H2AX detected in irradiated samples was especially high after irradiation with 12 and 16 Gy. Dose dependent increase of apoptosis was detected 48 hour after irradiation by flow-cytometry, with the maximum value of 20.4% after irradiation with 16 Gy, and the apoptotic index of 9.3. Pro-apoptotic effects of carbon ion beams were confirmed by changes of key molecules of the mitochondrial apoptotic pathway, p53 protein expression, Bax/Bcl-2 ratio and caspase-3 activation.

Key words: melanoma, carbon ion, apoptosis, p53, Bax/Bcl-2 ratio, caspase-3

INTRODUCTION

Human melanoma represents one of the major health problems. Approximately 11 million people worldwide are diagnosed with cancer, while each year almost 7 million die of the disease [1]. Although melanoma is known to be radio-resistant with powerful metastatic potential and dissimilar response to conventional radiation [2], about 40% of cured patients were exposed to different radiotherapeutic protocols. The aim of contemporary radiotherapy is to deliver the highest radiation dose to the tumour volume while sparing the surrounding healthy tissues as much as possible [3].

With respect to conventional radiotherapy, heavy ion beams have unique advantage due to their physical properties. Accelerated high-energy charged particles lose their energy through interactions with atoms in irradiated tissue, releasing large amount of energy at the end of range. The energy loss per unit of length along the particle track, which is the linear energy transfer or LET, is at the origin of induced biological effects. The value of LET depends on the particle species and their energy. It was shown that for carbon ions the LET values of ~ 200 keV/ μ m produce the larg-

est biological effectiveness. Even with this intermediate LET values, carbon ions compared to γ -rays or protons are able to better eliminate radio-resistant tumour cells [4, 5].

Ionizing radiation initiates diverse biochemical processes that determine cellular response. DNA is the most important target for ionizing radiation. Thus, exposure to radiation results directly in DNA injuries or indirectly in generation of chemically active free radicals that can, in turn, damage the molecular structure, resulting in cellular dysfunctions or mutations [6]. The principal lesions induced by ionizing radiation are DNA double-strand breaks (DSB). An early event after induction of DSB is phosphorylation of histone H2AX at serine 139 and formation of γ H2AX. The level of γ H2AX is closely correlated with the level of DSB and the cell death. If DSB were left unrepaired, they would trigger cell cycle arrest and/or apoptosis [7].

Apoptosis is one of the mechanisms of cancer cell death in response to chemotherapy and radiotherapy. A huge number of proteins are involved in the regulation of apoptosis. Interactions among pro-apoptotic members of the Bcl-2 family and death-promoting members like Bax and p53 have been suggested as the most important process in apoptosis [8]. The tumour suppressor protein p53 is the key regulator of apoptosis and carcinogenesis. It has a low expression level in normal

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cells and plays a protective role of genome in response to the variety of stresses with potential cancer risk. It can prevent cancer by provoking either cell cycle arrest or apoptotic cell death [9]. Mutations in the p53 are found to be present in most tumor types. Once the tumor suppressor becomes inactive, uncontrolled proliferation and abnormal increase in cell number can lead to cancer [10]. In the presence of death signal the p53 directly or indirectly activates the Bax protein which can promote changes in the permeability of the mitochondrial membrane [8, 11]. This event results in the release of cytochrome c and other pro-apoptotic factors from mitochondria [12]. It has been shown that after an apoptotic stimulus, proapoptotic members of Bcl-2 family are subjected to conformational changes and oligomerization and they cause mitochondrial outer membrane permeabilization [13]. The result of this permeabilization is the release of cytochrome c into the cytoplasm, which induces the formation of the Apaf1-containing macromolecular complex called the apoptosome. This complex, in turn, binds and activates procaspase-9. Mature caspase-9 remains bound to the apoptosome, recruiting and activating executioner caspase-3 and/or caspase-7. Caspase-3 is an effector caspase, which activity leads to the proteolytic degradation of substrates, resulting in the apoptotic morphology [14].

Unlike other human malignancies mutations in p53 gene are very rare in melanoma, [15]. Despite fully functional p53 melanoma cells can avoid apoptosis by inactivating downstream component of apoptotic pathway [16, 17]. Moreover, some molecules involved in the regulation of p53, such as nitric oxide (NO), are deregulated in melanoma. Human melanoma cells are known to constitutively express the enzyme, inducible nitric oxide synthase (iNOS), which is responsible for cytokine induced production of NO [18]. Furthermore, other signaling pathways are involved in regulation of melanoma cell death and survival, such as NF- κ B signaling. Namely, melanoma cells have elevated basal level of NF- κ B transcription factor that is involved in cell proliferation [19].

Efficiency of high LET ionizing radiation was already analyzed on a variety of human radio-sensitive cell lines, such as CAL4 melanoma, SCC25 oral squamous cell carcinoma, HepG2 hepatocellular carcinoma cells, *etc.* [20-22]. Same analysis was also done on some radio-resistant Bcl-2 over-expressing tumour cells [23]. The aim of this study was to investigate the behaviour of the human HTB140 melanoma cells that represent the limit case of cellular radio-resistance [24, 25]. Analysis of the effects of carbon ions were performed as they are considered to be effective even on radio-resistant cells [26]. Efficiency of carbon ions was estimated by their ability to induce DSB and apoptosis. Since the resistance of melanoma cells is mostly caused by the defect of apoptotic pathways, although they are usually p53+, the level of regulatory

and effector molecules involved in this process was also investigated.

MATERIALS AND METHODS

Cell culture

The human melanoma HTB140 cells were cultured in the RPMI1640 medium supplemented with 10% foetal bovine serum and penicillin/streptomycin (all from Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Cells were grown under standard conditions in a 5% CO₂ humidified incubator at 37 °C (Heraeus, Hanau, Germany).

Irradiation conditions

Analyses of the human HTB140 melanoma cells were performed within the Bragg curve of the 62 MeV/u ¹²C ion beam produced by the superconducting cyclotron at the Istituto Nazionale di Fisica Nucleare, Laboratori Nazionali del Sud, Catania, Italy. The doses ranged from 2 to 16 Gy with the average dose rate of 12 Gy/min. Irradiation position, as shown in fig. 1, was obtained by interposing 7.9 mm thickness Perspex plate (Polymethyl methacrylate – PMMA) in front of the cell monolayer. The corresponding relative dose was 73.4%, with the LET value of ~258 keV/μm. Reference dosimetry was performed using plane-parallel PTW 34045 Markus ionization chamber calibrated according to the international atomic energy agency (IAEA) code of practice [27, 28]. Since the range of the 62 MeV/u carbon ions is short, with a very narrow Bragg peak, the precision of positioning of cells was checked by placing GafChromic HS films (ISP Technologies, Wayne, N. J., USA) before each irradiation of the samples. Cell monolayer was fixed vertically in a special device, facing the horizontal beam. Gamma rays from a ⁶⁰Co source at the Vinča Institute of Nu-

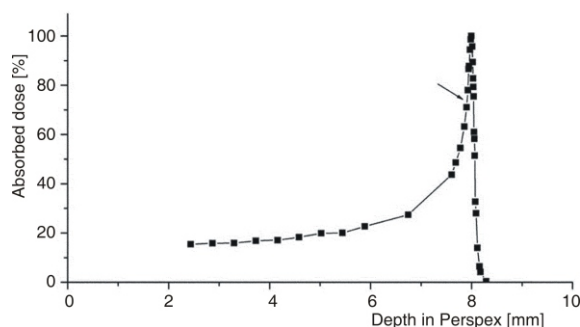


Figure 1. Depth dose distribution in Perspex of the 62 MeV/u ¹²C ion beam produced at INFN-LNS. Arrow indicates irradiation position corresponding to LET value of 258 keV/ m

clear Sciences in Belgrade were used as a reference radiation. All irradiations were carried out in air at room temperature.

Flow cytometric analyses

The flow cytometric analyses with Annexin V/propidium iodide (PI) double staining or only with PI were used for the evaluation of apoptosis of the HTB140 cells.

For quantification of apoptosis with Annexin V-FLUOS Staining kit (Roche Diagnostics GmbH, Mannheim, Germany) cells were trypsinized, washed in ice-cold phosphate-buffered saline (PBS) and resuspended in 100 μ l Annexin V FLUOS labeling solution (containing Annexin V – Fluos labeling reagent and PI). After incubation for 30 min in the dark, 500 μ l of incubation buffer was added per sample. Samples were analyzed on a FACSCalibur (Becton Dickinson, Heidelberg, Germany). For each sample, 10000 cells were analyzed. The number of apoptotic cells was calculated using ModFit software (Verity Software, Becton Dickinson).

For flow cytometric analysis with PI, the cells were collected by trypsinization and centrifuged for 10 min at 420 g. Then, they were washed with phosphate-buffered saline (PBS), fixed with cold 70% ethanol overnight, and resuspended in staining solution containing 50 μ g/ml PI (Sigma-Aldrich Chemie GmbH) and 50 μ g/ml Ribonuclease A (Promega Limited, Southampton, UK) in PBS buffer. After incubation at the room temperature for 30 min, 10000 cells per sample were analyzed by the flow cytometry (FACSCalibur). Percentage of apoptotic cells in sub-G1 phase of the cell cycle was calculated according to the ModFit software.

Western blot analysis

Total proteins for the Western blot analysis were extracted from the non-irradiated and irradiated HTB140 cells. Confluent cells were collected, washed with PBS and homogenized with buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% Nonidet P-40 (NP-40), 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium-deoxycholate, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 1 mM Na_3VO_4 , 5 g/mL aprotinin, 5 g/mL antipain, 5 g/mL leupeptin, 0.5 mM phenylmethanesulphonylfluoride (PMSF). For the analysis of γ H2AX lysis buffer additionally contained NaF, β -diglicophosphate and sodium-pirophosphate. The homogenate was centrifuged at 12900 g for 20 min at 4 °C. In the supernatant containing the whole-cell lysate the amount of proteins was quantified spectrophotometrically

using the Lowry method [29]. The samples were mixed with denaturizing buffer according to Laemmli [30] and boiled for 5 min. For the analysis of p53, Bax, and Bcl-2, 20 g of proteins were loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and electro-transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P membrane, Millipore, Bedford, Mass., USA) using a blot system (Transblot, BioRad, Hercules, Cal., USA). For the analysis of γ H2AX, 60 μ g of proteins were loaded onto a 12% SDS-PAGE. Membranes were blocked with PBS/milk (5% non-fat dry milk in PBS) for 1 hour at room temperature and then incubated at 4 °C overnight with 1:1000 dilution of the primary anti-p53, anti-Bax, anti-Bcl-2 antibodies (Cell Signaling, Danvers, Mass., USA) and anti- γ H2AX (BioLegend, San Diego, Cal., USA). For normalization purposes, immunoblots were incubated overnight at 4 °C with rabbit polyclonal anti- β -actin antibody (Sigma-Aldrich Chemie GmbH) diluted 1:1000 in PBS Tween 20 (PBST)/milk. After three washings in PBST, lasting for 10 min each, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit antibody (HRP, Cell Signaling) diluted 1:3000 in PBST/milk. After three washings with PBST, the proteins were visualized with an enhanced chemiluminescence (Sigma-Aldrich Chemie GmbH) and exposed to X-ray film. Protein molecular mass standards (PageRuler Plus Prestained Protein Ladder, Fermentas, Vilnius, Lithuania) were used for calibration. Densitometry of protein bands on X-ray film was performed using Image J Analysis PC software.

Caspase-3 activity assay

Caspase-3 activity was assessed using the Caspase-3 Colorimetric Assay Kit (Sigma Aldrich Chemie GmbH), following the manufacturer's instructions. This assay is based on the detection of the amount of Ac-DEVD-p-NA substrate cleaved by cell lysates to release the colored p-NA molecule. The assay was performed in 96-well plates.

Forty eight hours after irradiation $\sim 10^7$ cells were centrifuged at 660 g, cell pellets were washed in PBS and resuspended in lysis buffer (50 mM HEPES pH 7.4, 5 mM CHAPS, 5 mM DTT). Lysed cells were centrifuged at 15300 g and supernatants were used for further analysis. Equal amounts of proteins from each sample were added to wells containing the assay buffer (20 mM HEPES pH 7.4, 0.1% CHAPS, 5 mM DTT, 2 mM EDTA), followed by 10 l of Ac-DEVD-p-NA (20 mM), bringing the total volume of each well to 100 l. Caspase-3 activity was assessed by measuring optical density at 405 nm using the enzyme-linked immunosorbent assay (ELISA) reader (Wallac, Victor2 1420 Multilabel counter, Turku, Finland).

Statistical analysis

Data are presented as the Mean \pm SEM (standard error of the mean). Duplicate measurements were made during each experiment and all experiments were repeated at least three times. The significance of differences between the experimental groups was assessed by the independent Student's *t*-test. The level of significance was set at $p < 0.05$.

RESULTS

Ability of carbon ions to induce DSB was evaluated by the detection of γ H2AX using Western blot analysis. As shown in fig. 2 significant level of expression of γ H2AX was demonstrated in irradiated samples, both after γ -rays or ^{12}C ions. The effect was more pronounced after exposure of cells to carbon ions. Level of phosphorylated H2AX in control sample was minor. Especially high intensity of γ H2AX bands was detected in samples irradiated with higher doses of carbon ions (8, 12, and 16 Gy).

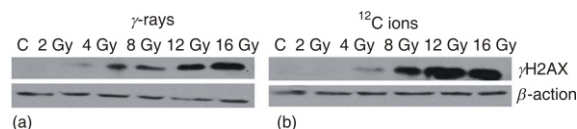


Figure 2. Western blot analysis of γ H2AX protein expression 2 h after exposure of HTB140 cells to γ -rays (a) and ^{12}C ions (b). Irradiation doses were 2-16 Gy

The percentage of apoptotic cells was evaluated by flow-cytometry, 48 hour after irradiation with γ -rays or ^{12}C ions. Gamma rays produced negligible quantity of apoptotic cells. The obtained dose dependent apoptosis in the HTB140 cells irradiated with carbon ions ranged from 5.3 to 20.4% and are presented in fig. 3.

The corresponding apoptotic indexes were calculated and are given in tab. 1. The maximum apoptotic index measured by PI staining was 7.7, while 9.3 when quantified by double Annexin V/PI staining, and were obtained after irradiation of cells with 16 Gy carbon ions. The acquired results clearly indicate the ability of ^{12}C ions to provoke the apoptotic cell death of the resistant HTB140 cells in the whole range of the doses applied.

To examine the molecular level of apoptosis, key regulatory molecules of the mitochondrial apoptotic pathway were followed under the described experimental conditions. The level of the p53 protein expression was assessed and is presented in fig. 4(a). The dose dependent increase in the expression of p53 protein ranged from 238 to 554% and confirmed the induction of apoptosis fig. 4(a). Major boost was achieved with 12 and 16 Gy ($p < 0.001$, ***).

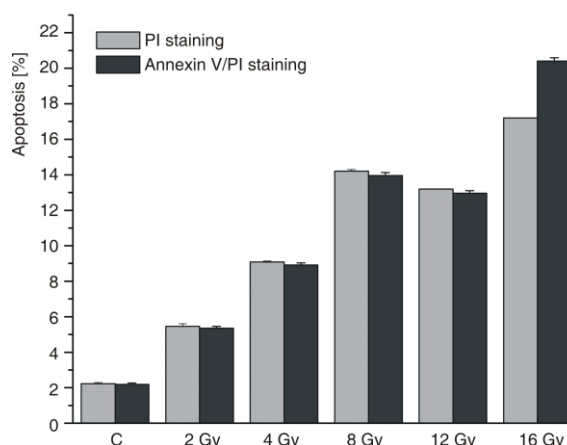


Figure 3. The dose dependent apoptosis in the HTB140 cells 48 h after irradiation with ^{12}C ions (a). Irradiation doses were 2-16 Gy. Number of apoptotic cells was determined by flow cytometric analyses using PI staining or double Annexin V-FLUOS/PI staining

Table 1. Apoptotic indexes of the HTB140 cells 48 h after irradiation with ^{12}C ions

Dose [Gy]	0	2	4	8	12	16
Apoptotic index (AI) (PI staining)	1	2.45	4.07	6.37	5.92	7.71
Apoptotic index (AI) (Annexin V/PI staining)	1	2.35	4.55	8.70	6.78	9.31

The expression of the Bax and Bcl-2 regulatory proteins, presented as the ratio of Bax and Bcl-2 is given in fig. 4(b). With the rise of the radiation dose the level of Bax increased while, the level of Bcl-2 protein decreased. The dose dependent increase of Bax/Bcl-2 ratio from 1.04 to 3.08 was estimated with a statistical significance for 8 Gy ($p < 0.05$, *), 12 and 16 Gy ($p < 0.01$, **).

In order to acquire more details about the mechanism of apoptosis, the caspase-3 activity, as one of key effector apoptotic molecules, was followed as well and is given in fig. 5. The level of caspase-3 activity of untreated HTB140 cells was set at a nominal value of 1 and was compared with the values of caspase-3 activity in HTB140 cells that were exposed to ^{12}C ions. Irradiation led to the significant increase of the caspase-3 activity for 53-88% ($p < 0.001$, ***), with the best effect achieved after irradiation with 16 Gy.

DISCUSSION

Human melanoma is one of the most aggressive forms of cancer with rapidly growing incidence. Nowadays, great efforts are made to overcome this disease. Contemporary treatments to sterilize melanoma are

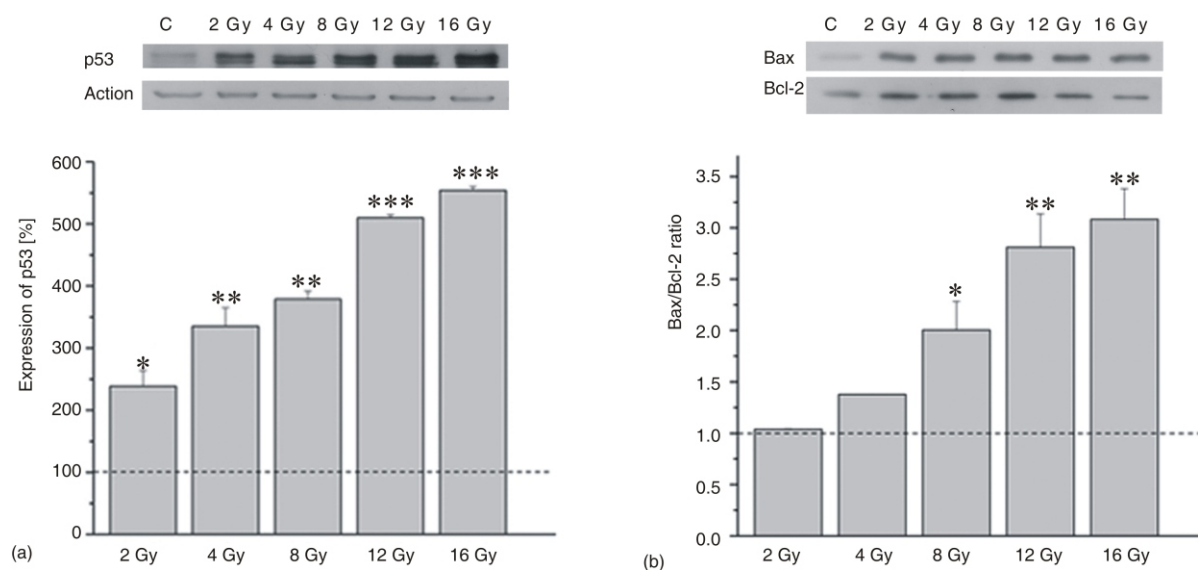


Figure 4. Western blot analysis of p53 protein expression (a) and Bax/Bcl-2 ratio (b) 48 h after irradiation of HTB140 cells with ^{12}C ions. Irradiation doses were 2-16 Gy, * – $0.01 < p < 0.05$, ** – $0.001 < p < 0.01$, and *** – $p < 0.001$

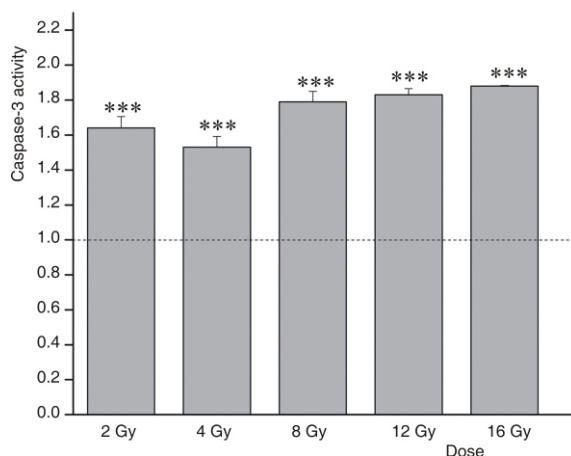


Figure 5. Caspase-3 activity detected by colorimetric assay 48 h after irradiation of HTB140 cells ^{12}C ions. The level of caspase-3 activity of control cells was set at a nominal value of 1 and the values of caspase-3 activity in irradiated samples were compared to this value. Irradiation doses were 2-16 Gy, *** – $p < 0.001$

surgery, radio- and chemotherapy [31]. Efficiency of therapy depends on the sensitivity of melanoma cells. In our previous studies radio-sensitivity of two melanoma cell lines, HTB140 and HTB63, was assessed. The obtained results indicated that these two cell lines represent the opposite end-points on the scale of cellular radio-sensitivity. The HTB140 cells are among the most radio-resistant with the value of the surviving fraction at 2 Gy (SF2) of 0.961 for γ -rays, while the HTB63 cells have the SF2 value of 0.061 and are very radio-sensitive [24]. Considering that for the HTB63 cells SF2 value as well D_{10} (the dose that reduces survival from 1 to 0.1) of 1.7 Gy are already low [24], it was meaningless to use this cell line for the analysis of the effects of highly efficient radiation like carbon ions.

It is known that anticancer agents mostly act as inducers of apoptotic cell death [32, 33]. In the past induction of apoptosis after exposure to high ionizing radiation was analyzed on different radio-sensitive and radio-resistant cell lines [22, 23, 34].

The aim of this study was to investigate the induction of DSB and the level of apoptosis in the highly radio-resistant human HTB140 cell line after irradiations with carbon ions. Gamma rays were used as the reference radiation. Literature data suggested that optimal time for the detection of DSB is up to a few hours after irradiation [34, 35]. Therefore the analysis of γH2AX was performed 2 hour after irradiation. Pro-apoptotic ability was analyzed 48 hour after irradiation. This time point was chosen because in the majority of cells, apoptosis does not occur within the first 24 hour after irradiation but arises after one or more cell divisions. The maximal number of apoptotic cells in *in vitro* conditions can be found 48 hour after irradiation, especially for radio-resistant cells [36]. Also, in our previous kinetic studies the inhibition of the HTB140 cell growth was extensively investigated after different treatments with proton radiation or alkylating agents (fotemustine, FM and dacarbazine, DTIC), as well as after their combined application. Irradiations with protons were performed at the full energy Bragg peak maximum and along the therapeutic proton spread out Bragg peak (SOBP) [24, 25]. Protons provoked dose dependent elimination of the HTB140 cells 6, 24, 48 hours, and 7 days post-irradiation. Also, a wide range of FM and DTIC concentrations has been analyzed. The effects of FM and DTIC (0.05-2 mM) on cell viability and proliferation were investigated in a time dependent manner, *i. e.*, after 6, 24, 48, 72, 96 hours, and 7 days. The best elimination of the HTB140 cells was achieved 48 hours after treat-

ment with FM and DTIC [37]. In addition, apoptotic cell death was evaluated 6, 12, 24 or 48 hours after treatments with dexamethasone used as a positive control, as well as with FM, DTIC, γ -rays and protons. The best induction of apoptosis in the HTB140 cells was achieved 48 hours after treatment with drugs and/or protons [38, 39].

Effects of carbon ions on HTB140 cells were analyzed for a broad spectrum of LET values, from ~ 82 -742 keV/ μ m. The best induction of apoptosis was obtained after irradiation with 258 keV/ μ m [40, 41]. Since this LET value is close to those reported by other authors for the analysis of the effects of carbon ions on malignant cells *in vitro* [33], we have chosen it for the further analysis of pro-apoptotic ability of carbon ions.

Considering that DSB are critical lesions induced by radiation, efficiency of carbon ions *vs.* conventional γ -rays was estimated by the level of phosphorylation of H2AX, as the consequence of DSB. Obtained results indicated that carbon ions induced DSB to a greater extent than γ -rays. This suggests better antitumour efficiency of the high LET radiation. Especially strong signal of γ H2AX was detected after irradiation with higher doses. The level of γ H2AX in control samples and samples exposed to 2 Gy irradiation was very low and impossible to detect by Western blot. Literature data suggested that immunofluorescent microscopy would be a more appropriate method for the analysis of lower doses [42]. However, since we analyzed a wide range of doses, Western blot was selected as a suitable method. It is known that γ H2AX is essential for concentrating repair proteins and maintaining the integrity of the DNA [43]. If the DNA DSB are not repaired, injured cells will go to apoptosis [44]. Since obtained results indicate that γ -rays induced lower level of γ H2AX, as well as apoptosis [24], it could be assumed that DSB induced by low LET radiation are more repairable than those induced by high LET radiation. Based on these data, analysis of apoptotic pathways in HTB140 cells was performed only after irradiation with carbon ions.

Carbon ions induced the dose dependent increase of apoptotic nuclei 48 hours after irradiation of the HTB140 cells. Similar level of apoptosis was already reported for the same cell line exposed to protons [24], while on the contrary γ -rays induced a very small number of apoptosis [45]. All these results are in agreement with very high level of radio-resistance of the HTB140 cells [24, 25]. Moreover, this level of apoptosis was in the range that has already been reported for other resistant cell lines, such as FRTL-5, in which apoptosis was induced by different radiation sources [46]. The dose dependent increase of apoptotic cells could be attributed to the severe damage induced by high LET of carbon ions, leading to the induction of late apoptosis or necrosis.

In order to corroborate the induction of apoptosis, the analysis of the regulatory proteins in-

involved in this process was performed. The obtained results imply that the apoptosis induced with carbon ions are correlated with the increased p53 expression. Moreover, the Bax/Bcl-2 ratio was shifted in favor of apoptosis. The inactivation of the p53 tumor suppressor was detected in over half of human tumors [47]. In the response to cellular stress, such as DNA damage induced by drugs or radiation, the p53 tumor suppressor protein is able to mediate cell cycle arrest as well as apoptosis [48, 49]. Low levels of DNA damages activate ATM (*ataxia telangiectasia mutated*) protein, which in turn triggers phosphorylation of p53. Upon phosphorylation, p53 becomes stabilized and blocks proliferation by upregulation of p21, which triggers G1/S arrest. With high levels of DNA damages, however, p53 can activate pro-apoptotic Bcl-2 family members, such as Bax and, thereby, causes the release of cytochrome c from mitochondria. The mechanism by which p53 protein might trigger apoptotic machinery involves transcriptional activation of the pro-apoptotic members of the Bcl-2 family and repression of the anti-apoptotic regulators [10]. The Bcl-2 is a large family of proteins that includes pro-apoptotic members such as Bax and Bad, as well as the anti-apoptotic proteins Bcl-2, Bcl-XL and Bcl-w. Activated pro-apoptotic members of the Bcl-2 family neutralize the anti-apoptotic members of the same family [50]. Without this inactivation, anti-apoptotic proteins would inhibit cell death [51]. Therefore, the balance between the levels of Bax and Bcl-2 proteins is very important for the determination of the apoptotic response. On the other hand, some authors pointed out other signaling pathways involved in the regulation of melanoma development, such as RAS-RAF-MEK-ERK and p16INK4A-cyclinD-CDK4/6-RB. Simultaneous inhibition of MEK and CDK4 signaling molecules could induce apoptosis in melanoma cells [52]. Also, it has been shown that heavy ions could induce apoptosis independently of p53 status [53].

The obtained caspase-3 results confirm the above assertion. Caspase-3 is an effector caspase that leads to the proteolytic degradation of substrates, resulting in the apoptotic morphology [54]. The irradiation with carbon ions obviously up-regulated the caspase-3 activity, that is in agreement with the results of other authors [55].

CONCLUSION

Taking into account the obtained results it is clear that carbon ions induce DSB as well as apoptosis in the HTB140 melanoma cells. However, for such radio-resistant cells, it will be necessary to further improve therapeutic outcomes. The future aspect in overcoming the high radio-resistance of melanoma might be the application of combined treatments using radiation and chemical agents that are capable to selectively decrease the level of the anti-apoptotic molecules in cells [56].

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AUTHOR CONTRIBUTIONS

Experiments were designed by A. Ristić-Fira and I. Petrović, and performed by all authors. Irradiations and dosimetry were carried out by I. Petrović, A. Ristić-Fira, P. Cirrone, and G. Cuttone, while biological assays were done by A. Ristić-Fira, L. Korićanac, J. Žakula, and O. Keta. Data analyses and discussion were accomplished by A. Ristić-Fira, L. Korićanac, and I. Petrović. The manuscript was written by L. Korićanac and J. Žakula, and supervised by A. Ristić-Fira and I. Petrović. Figures were prepared by L. Korićanac, J. Žakula, and O. Keta.

REFERENCES

- [1] Anand, P., *et al.*, Cancer is a Preventable Disease that Requires Major Lifestyle Changes, *Pharm Res.*, 25 (2008), 9, pp. 2097-2116
- [2] Barranco, S. C., Romsdahl, M. M., Humphrey, R. M., The Radiation Response of Human Malignant Melanoma Cells Grown *in vitro*, *Cancer Res.*, 31 (1971), 6, pp. 830-833
- [3] Dosanjh, M., Development of Hadron Therapy for Cancer Treatment in Europe, in: Eds. G. Herrera Corral, L. M. Montano Zetina, Medical Physics Tenth Symposium on Medical Physics, American Institute of Physics, Mexico City, Mexico, 2008, pp. 12-16
- [4] Ando, K., *et al.*, Biological Gain of Carbon-Ion Radiotherapy for the Early Response of Tumor Growth Delay and Against Early Response of Skin Reaction in Mice, *J. Radiat. Res.*, 46 (2005), 1, pp. 51-57
- [5] Pommier, P., *et al.*, Particle Therapy: Carbon Ions, *Bull. Cancer*, 97 (2010), 7, pp. 819-829
- [6] Agrawal, A., Goyal, P. K., Radiation-Induced Hematological Alterations and Their Inhibition by Aegle Marmelos Frut Extract, *Nucl Technol Radiat*, 26 (2011), 3, pp.237-244
- [7] Muslimović, A., *et al.*, Numerical Analysis of Etoposide Induced DNA Breaks, PLoS ONE, 2009, doi: 10.1371/annotation/290cebfd-d5dc-4bd2-99b4-f4cf0be6c838
- [8] Fulda, S., Debatin, K. M., Extrinsic Versus Intrinsic Apoptosis Pathways in Anticancer Chemotherapy, *Oncogene*, 25 (2006), 34, pp. 4798-4811
- [9] Lane, D. P., Cancer. p53, Guardian of the Genom,e *Nature*, 358 (1992), 6381, pp. 15-16
- [10] Thornborrow, E. C., *et al.*, A Conserved Intronic Response Element Mediates Direct p53-Dependent Transcriptional Activation of Both the Human and Murine Bax Genes, *Oncogene*, 21 (2002) 7, pp. 990-999
- [11] Gross, A., *et al.*, Enforced Dimerization of BAX Results in Its Translocation, Mitochondrial Dysfunction and Apoptosis, *EMBO J.*, 17 (1998) 14, pp. 3878-3885
- [12] Gross, A., McDonnell, J. M., Korsmeyer, S. J., BCL-2 Family Members and the Mitochondria in Apoptosis, *Genes. Dev.*, 13 (1999), 15, pp. 1899-1911
- [13] Miyashita, T., *et al.*, Tumor Suppressor p53 is a Regulator of bcl-2 and Bax Gene Expression *in vitro* and *in vivo*, *Oncogene*, 9 (1994), 6, pp. 1799-1805
- [14] D'Amelio, M., Cavallucci, V., Cecconi, F., Neuronal Caspase 3: Not Only Cell Death, *Cell Death Differ.*, 17 (2010), 7, pp. 1104-1114
- [15] Sauroja, I., *et al.*, Analysis of G1/S Checkpoint Regulators in Metastatic Melanoma, *Genes Chromosomes Cancer*, 28 (2000), 4, pp. 404-414
- [16] Satyamoorthy, K., Bogenrieder, T., Herlyn, M., No Longer a Molecular Black Box-New Clues to Apoptosis and Drug Resistance in Melanoma, *Trends Mol. Med.*, 7 (2001), 5, pp. 191-194
- [17] Box, N. F., Terzian, T., The Role of p53 in Pigmentation, Tanning and Melanoma, *Pigment Cell Melanoma Res.*, 21 (2008), 5, pp. 525-533
- [18] Ekmekcioglu, S., *et al.*, Inducible Nitric Oxide Synthase and Nitrotyrosine in Human Metastatic Melanoma Tumors Correlate with Poor Survival, *Clin. Cancer Res.*, 6 (2000), 12, pp. 4768-4775
- [19] Gray-Schopfer, V. C., *et al.*, Tumor Necrosis Factor-Alpha Blocks Apoptosis in Melanoma Cells when BRAF Signaling is Inhibited, *Cancer Res.*, 67 (2007), 1, pp. 122-129
- [20] Courdi, A., *et al.*, The Depth-Dependent Radiation Response of Human Melanoma Cells Exposed to 65 MeV Protons, *Brit. J. Radiol.*, 67 (1994), pp. 800-804
- [21] Bettega, D., *et al.*, Radiobiological Studies on the 65 MeV Therapeutic Proton Beam at Nice Using Human Tumour Cells, *Int. J. Radiat. Biol.*, 76 (2000), 10, pp. 1297-1303
- [22] Lee, K. B., *et al.*, Low Energy Proton Beam Induces Tumor Cell Apoptosis through Reactive Oxygen Species and Activation of Caspases, *Exp. Mol. Med.*, 40 (2008), 1, pp. 118-229
- [23] Mori, E., *et al.*, High LET Heavy Ion Radiation Induces p53-Independent Apoptosis, *J. Radiat. Res.*, 50 (2009), 1, pp. 37-42
- [24] Petrović, I., *et al.*, Radiobiological Analysis of Human Melanoma Cells on the 62 MeV CATANA Proton Beam, *Int. J. Radiat. Biol.*, 82 (2006), 4, pp. 251-265
- [25] Petrović, I., *et al.*, Response of a Radioresistant Human Melanoma Cell Line Along the Proton Spread-out Bragg Peak, *Int. J. Radiat. Biol.*, 86 (2010), 9, pp. 742-751
- [26] Hofman-Hüther, H., *et al.*, Induction of Reproductive Cell Death and Chromosome Aberrations in Radioresistant Tumour Cells by Carbon Ions, *Int. J. Radiat. Biol.*, 80 (2004), 6, pp. 423-435
- [27] Cirrone, G. A. P., *et al.*, A 62 meV Proton Beam for the Treatment of Ocular Melanoma at Laboratori Nazionali del Sud – INFN, *IEEE Trans. Nuc. Sci.*, 51 (2004), 3, pp. 860-865
- [28] ***, Absorbed Dose Determination in External Beam Radiotherapy: an International Code of Practise for Dosimetry Based on Standards of Absorbed Dose to Water, IAEA Technical Report Series N, 398, 2000, pp. 135-150
- [29] Lowry, O. H., *et al.*, Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem.*, 193 (1951), 1, pp. 265-275
- [30] Laemmli, U. K., Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4, *Nature*, 227 (1970), 5259, pp. 680-685
- [31] Soengas, M., Lowe, S., Apoptosis and Melanoma Chemoresistance, *Oncogene*, 22 (2003), 20, pp. 3138-3151

- [32] Nicholson, D. W., From Bench to Clinic with Apoptosis-Based Therapeutic Agents, *Nature*, 407 (2000), 6805, pp. 810-816
- [33] Tamm, I., Schriever, F., Dörken, B., Apoptosis: Implications of Basic Research for Clinical Oncology, *Lancet Oncol.*, 2 (2001), 1, pp. 33-42
- [34] Ghosh, S., *et al.*, DNA Damage Response Signaling in Lung Adenocarcinoma A549 Cells Following Gamma and Carbon Beam Irradiation, *Mutat. Res.*, 716 (2011), 1-2, pp. 10-19
- [35] Attardi, L., de Vries, A., Jacks, T., Activation of the p53-Dependent G1 Checkpoint Response in Mouse Embryo Fibroblasts Depends on the Specific DNA Damage Inducer, *Oncogene*, 23 (2004), 4, pp. 973-980
- [36] Bernhard, E. J., *et al.*, Reducing the Radiation-Induced G2 Delay Causes HeLa Cells to Undergo Apoptosis Instead of Mitotic Death, *Int. J. Radiat. Biol.*, 69 (1996), 5, pp. 575-584
- [37] Ristić-Fira, A. M., *et al.*, Assessment of the Inhibitory Effects of Different Radiation Qualities or Chemotherapeutic Agents on a Human Melanoma Cell Line, *Phys. Med.*, 24 (2008), 4, pp. 187-195
- [38] Ristić-Fira, A. M., *et al.*, Effects of Fotemustine or Dacarbazine on a Melanoma Cell Line Pretreated with Therapeutic Proton Irradiation, *J. Exp. Clin. Cancer Res.*, (2009), doi: 10.1186/1756-9966-28-50
- [39] Ristić-Fira, A., *et al.*, Proton Inactivation of Melanoma Cells Enhanced by Fotemustine, *Radiat. Prot. Dosimetry*, 143 (2011), 2-4, pp. 503-507
- [40] Žakula, J., *et al.*, Apoptosis of HTB140 Melanoma Cells Induced by Carbon Ions of Different LET, *LNS Activity Report 2009* (2010), pp. 238-241
- [41] Petrović, I., *et al.*, Melanoma Cells along a Carbon Ion Bragg Curve, *LNS Activity Report 2010* (2011), pp. 241-244
- [42] Desai, N., *et al.*, Immunofluorescence Detection of Clustered γ H2AX Foci Induced by HZE-Particle Radiation, *Radiat. Res.*, 164 (2005), pp. 518-522
- [43] Fragkos, M., Jurvansuu, J., Beard, P., H2AX is Required for Cell Cycle Arrest via the p53/p21 Pathway, *Mol. Cell. Biol.*, 29 (2009), 10, pp. 2828-2840
- [44] Okayasu, R., Repair of DNA Damage Induced by Accelerated Heavy Ions – a Mini Review, *Intern. J. Cancer*, 130 (2012), 5, pp. 991-1000
- [45] Ristić-Fira, A. M., *et al.*, Response of a Human Melanoma Cell Line to Low and High Ionizing Radiation, *Ann. N.Y. Acad. Sci.*, 1095 (2007), pp. 165-174
- [46] Green, L. M., *et al.*, Response of Thyroid Follicular Cells to Gamma Irradiation Compared to Proton Irradiation, I. Initial Characterization of DNA Damage, Micronucleus Formation, Apoptosis Cell Survival, and Cell Cycle Phase Redistribution, *Radiat. Res.*, 155 (2001), pp. 32-42
- [47] Hollstein, M., p53 Mutations in Human Cancers, *Science*, 253 (1991), 5015, pp. 49-53
- [48] Mercer, W. E., *et al.*, Negative Growth Regulation in a Glioblastoma Tumor Cell Line that Conditionally Expresses Human Wild-Type p53, *Proc. Natl. Acad. Sci. USA*, 87 (1990), 16, pp. 6166-6170
- [49] Meek, D. W., Tumour Suppression by p53: a Role for the DNA Damage Response? *Nat. Rev. Cancer*, 9 (2009), 10, pp. 714-723
- [50] Roos, W. P., Kaina, B., DNA Damage-Induced Cell Death by Apoptosis, *Trends Mol. Med.*, 12 (2006), 9, pp. 440-450
- [51] Hockenbery, D., *et al.*, Bcl-2 is an Inner Mitochondrial Membrane Protein that Blocks Programmed Cell Death, *Nature*, 348 (1990), 6299, pp. 334-336
- [52] Li, J., *et al.*, Simultaneous Inhibition of MEK and CDK4 Leads to Potent Apoptosis in Human Melanoma Cells, *Cancer Invest.*, 28 (2010), 4, pp. 350-356
- [53] Hamada, N., *et al.*, Energetic Heavy Ions Overcome Tumor Radioresistance Caused by Overexpression of Bcl-2, *Radiother. Oncol.*, 89 (2008), 2, pp. 231-236
- [54] Hunter, A. M., LaCasse, E. C., Korneluk, R. G., The Inhibitors of Apoptosis (IAP) as Cancer Targets, *Apoptosis*, 12 (2007), 9, pp. 1543-1568
- [55] Jin, X., *et al.*, Radiosensitization by Inhibiting Survivin in Human Hepatoma HepG2 Cells to High-LET Radiation, *J. Radiat. Res.*, 52 (2011), 3, pp. 335-341
- [56] Jansen, B., *et al.*, Chemosensitisation of Malignant Melanoma by BCL2 Antisense Therapy, *Lancet*, 356 (2000), 9243, pp. 1728-1733

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**ЈОНИ УГЉЕНИКА ИНДУКУЈУ ДВОЛАНЧАНЕ ПРЕКИДЕ ДНК И
АПОПТОЗУ У НТВ140 ЋЕЛИЈАМА ХУМАНОГ МЕЛАНОМА**

Ова студија спроведена је на радио-резистентним ћелијама НТВ140 хуманог меланом у циљу испитивања способности јона угљеника да индукују дволанчане прекиде на молекулу ДНК и апоптозу. Ћелије су озрачене јонима угљеника који имају линеарни трансфер енергије од 258 keV/ m. Примењене дозе зрачења биле су у опсегу од 2 до 16 Gy. Индукција дволанчаних прекида ДНК је праћена 2 сата након озрачивања, одређивањем нивоа γ H2AX протеина. Повећање нивоа γ H2AX протеина детектовано у озраченим узорцима посебно је изражено након излагања дозама од 12 и 16 Gy. Дозно зависно повећање броја апоптотских нуклеуса је детектовано методом проточне цитометрије 48 сати после озрачивања ћелија. Максимална вредност од 20,4% апоптозе и апоптотски индекс од 9,3 детектовани су након примене дозе од 16 Gy. Про-апоптотски ефекат јона угљеника је потврђен и праћењем промена кључних молекула митохондријалног апоптотског пута, као што су експресија p53 протеина, однос Bax/Bcl-2 протеина као и активација каспазе 3.

Кључне речи: меланом, јон угљеника, апоптоза, p53, однос Bax/Bcl2, каспаза-3
