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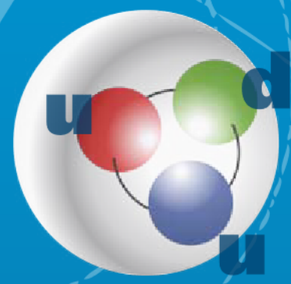
ACTIVITY REPORT 2008



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ACTIVITY REPORT 2008

LNS ACTIVITY REPORT 2008

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Cover figure:

High energy neutrino astronomy needs detector volumes of km^3 scale to reveal the rare neutrino events producing tracks in the detector medium. Neutrino telescopes have to be shielded from the atmospheric muon background that would completely swamp the tiny effects due to neutrinos. For this reason, such detectors are deployed in deep sea or ice that act as radiator of Cherenkov light and as a screen for muon background. At the South Pole the IceCube km^3 detector is under construction in deep antartic ice. A km^3-scale neutrino telescope in Mediterranean Sea, i.e. in the opposite hemisphere with respect to IceCube, will allow to achieve full coverage of the neutrino sky. In particular, a telescope in the Mediterranean Sea will allow to watch the Galactic Centre where interesting new phenomena were recently observed.
The construction of an underwater high energy neutrino telescope represents a remarkable challenge. Indeed, in deep sea instrumentation must be protected against corrosion and pressures as high as 350 bar. Moreover, the experiment reliability must be tuned to a life time of the order of ten years.
NEMO (NEutrino Mediterranean Observatory) is an advanced R&D project that aims at the design, realization and validation of key technologies needed for the construction, deployment and maintenance of an underwater high energy neutrino telescope in the Mediterranean Sea.

Many thanks are due to Giorgio Riccobene who provided the physics idea of the cover design

INFN-LNS n.11 (2009)

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I.Baran, C.Ganea, A.Scordino, F. Musumeci, S.Tudisco, S.Privitera, R.Grasso, V.Barresi, D.Condorelli, V.Baran, E.Katona, M.M.Mocanu, M.Gulino, M.Surcel, C.Ursaciu

General Information

INDUCTION OF APOPTOSIS BY FOTEMUSTINE, DACARBAZINE AND PROTONS IN A HUMAN MELANOMA CELL LINE

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Abstract

The effects of combined treatments with fotemustine (FM) or dacarbazine (DTIC) and proton irradiation on apoptotic cell death and *p53* gene expression, were investigated. The HTB 140 human melanoma cells were treated with 100 μM and 250 μM FM or DTIC and exposed to 12 and 16 Gy protons. All analyses were assessed 6 or 48 h after irradiation. The best effects on apoptosis and up-regulation of *p53* expression were obtained after combined treatment with higher FM concentration and protons, at 48 h after irradiation.

INTRODUCTION

Melanoma is an aggressive malignancy of melanocites. Currently, the subjects of ongoing melanoma research are risk factors that promote genesis of melanoma. Although primary cutaneous melanoma has a defined therapeutic approach and a high cure rate, metastatic melanoma is characterized by poor outcomes [1]. Even through considered as the most radioresistent tumour types [2], some forms of specially localized melanomas are curable nowdays. Thus, uveal melanoma and some other eye tumours are treated with therapeutic proton beam successfully [3]. This is due to the physical properties of proton beams, such as well-defined range, small lateral scattering, and high energy deposition at the precisely controled position, just before the end of the Bragg peak. For practical application, to cover the volume of the treated tumour, the spread out Bragg peak (SOBP) is used. It is obtained by the superposition of numerous monoenergetic peaks.

Although current treatments with chemoterapeutic drugs do not provide a significant therapeutic benefit only few citotoxyc drugs have been proven active against this tumour. Fotemustine (FM) is a member of the chloroethylnitrosurea class of alkylating agents, which gave encouraging results particulary in treatmant of metastatic melanoma [4]. Another commonly used drug dacarbazine (DTIC) is a monofunctional alkylating agent, which is frequently used in chemotherapy but usually resulting in partial tumour response, which lasts only for a few months [5].

Most chemotherapeutic agents realize their action in different cancer cells through induction of apoptosis. Small efficiency of these agents in the elimination of melanoma is the result of their inability to induce apoptosis in this type of tumour. The lack of apoptosis in some melanoma cell lines correlates with their high metastatic potential [6].

Increasing number of facts indicated that the initial signal that leads to the induction of apoptosis in melanoma cells must be increased by the transcription regulators, such as *p53* [7].

Regarding the importance that deregulation of apoptotic pathways have in tumour progression, the use of apoptosis as a biological phenomenon is one of the basic strategies in the formation of therapeutic approaches for treatment of various types of cancer [8].

Considering the significance that apoptosis has in the tumour development, as well as in the development of chemo- and radioresistance, in this study, the effects of single and combined treatments with alkylating agents and/or proton radiation on these processes were analyzed. For this purpose human melanoma cell line was used as a model system.

MATERIALS AND METHODS

Cell Culture

The human HTB 140 melanoma cells were cultured as a monolayer in the RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine and penicillin/streptomycin, in humidified atmosphere of 5 % CO_2 at 37°C.

Experimental Conditions

Cells were exposed to 100 μM and 250 μM fotemustine (FM, Ital Farmaco S.p.A., Milano, Italy) or dacarbazine (DTIC, Aventis Pharma S.p.A., Milan, Italy) 24 h after seeding. In combined treatments, cells were exposed to drugs 24 h after seeding and than to proton irradiation 24h later.

Irradiation with 62 MeV proton beam was performed at the CATANA (Centro di Adro Terapia e Applicazzioni Nucleari Avanzati) facility at INFN – LNS, Italy, with the

irradiation position in the middle of the SOBP. Delivered single doses were 12 and 16 Gy, at the dose rate of 15Gy/min. Effects of single and combined treatments on HTB 140 cells were assessed 6 or 48 h after irradiation.

Quantification of Apoptosis

Quantification of apoptotic and necrotic cells was performed using flow cytometric analyses with Annexin V-FLUOS Staining kit (Roche Diagnostics GmbH, Mannheim, Germany). Samples were analyzed on a fluorescence activated cell sorter (FACS) (Becton Dickenston, USA). The number of apoptotic and necrotic cells were calculated using a computer program CellQest.

RT PCR

The total RNAs were isolated using RNeasy Total Preparation kit (Quigen, UK) according to the instructions of manufacturer. The purity and quantity of the RNAs were determined spectrophotometrically and the samples were checked by agarose gel electrophoresis. Isolated RNAs were reverse transcribed to cDNAs using First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instruction. The products of PCR amplification were normalized against the "housekeeping" gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences purchased from MBI Fermentas, Vilnius, Lithuania and PCR product sizes were:

GAPDH, 5' CGGAGTCAACGGATTTGGTCGTAT 3'

5' AGCCTTCTCCATGGTGGTGAAGAC 3' (306 bp);

p53, 5' AGATAGCGATGGTCTGGC 3'

5' TTGGGCAGTGCTCGCTTAGT 3' (380 bp);

The PCR steps were carried out on a thermal cycler (Eppendorf, Hamburg, Germany). Thermocycling conditions included denaturation at 94°C for 4 min (one cycle), then denaturation at 94°C (45 s), annealing at 58°C (45 s) for p53 and 55°C (45 s) for GAPDH, extendion at 72°C (45 s) for 35 cycles and the final extension at 72ºC for 10 min (one cycle). Amplification products were analysed by electrophoresis at 2 % agarose gel. Quantification was performed using Multi-Analyst/PC Software Image Analysis System.

RESULTS

The ability of FM, DTIC and/or proton irradiation to induce apoptosis was observed 6 and 48 h after each single or combined treatment.

The effects of FM and protons on induction of apoptosis

Six hours after exposure to protons, the percentage of apoptotic cells in irradiated samples increased (15 % - 25%). Single treatment with FM also resulted in an increase of apoptotic nuclei (5.2 - 7.3 %). The combined treatment of HTB140 cells with FM and protons caused an increase in Annexin V positive population that ranged from 4 to 19 %.

Percentage of apoptotic nuclei in irradiated samples 48h after irradiation also increased as compared to control and ranged from 17.4 to 18.1 %. At the same time point the increase in apoptosis was also observed after the treatment with FM (9.8 - 12.1 %). Combined treatments with 100 μM FM and protons induced an increase in the percentage of apoptotic cells that ranged from 12 to 16 %. The highest level of induction of apoptosis was observed after the exposure of HTB 140 cells to 250 μm FM and protons and was 38 to 41 % (Table 1). Corresponding Apoptotic indexes are given in the Table 1.

Table 1: Apoptosis (%) and apoptotic index (AI) 6 and 48h after treatment with FM, DTIC and/or protons

	6h		**48h**	
	% of apoptosis	AI	% of apoptosis	AI
K	3.58	1	6.38	1
12 Gy	15.35	4.29	18.13	2.84
16 Gy	25.15	7.03	17.42	2.73
100 μM FM	7.28	2.03	9.85	1.54
250 μM FM	5.2	1.45	12.11	1.9
100 μM FM + 12 Gy	4	1.12	16	2.51
100μM FM + 16Gy	19	5.31	12	1.88
250μM FM + 12Gy	6	1.68	38	5.96
250μM FM + 16Gy	7	1.96	41	6.43
100μM DTIC	3.19	0.88	7.54	1.18
250μM DTIC	2.76	0.77	7.4	1.16
100μM DTIC + 12Gy	6	1.68	3	0.47
100μM DTIC + 16Gy	7	1.95	4	0.63
250μM DTIC + 12Gy	14	3.91	7	1.1
250μM DTIC + 16Gy	9	2.51	7	1.1

The analysis of *p53* gene expression, 6 h after single treatments has shown that 12 Gy proton irradiation and also single treatment with 100 μM FM have not induced significant changes as compared to the control. However, the dose of 16 Gy has stimulated the increase of *p53* gene expression for 15 % (***, $p<0.001$), while treatment with 250 μM FM increased the level of *p53* gene expression for 32 % (***, $p<0.001$). Combination of FM and protons has lead to an increase in the level of *p53* in the range of 15 to 28 %. Combined treatments with 100 μM FM and protons have shown better effect on the induction of gene expression as compared to the effect of single 100 μM FM (***, $p<0.001$). In irradiated cells that were previously treated with 250 μM FM, the level of *p53* was lower in comparison to the cells treated only with 250 μM FM ($p<0.05$) (Figure 1A).

The analysis of *p53* gene expression, 48 h after irradiation, has shown that proton irradiation and single treatment with FM did not cause significant changes as compared to the control. Irradiated cells pretreated with 100 μM FM exhibited a slight increase in the *p53*

expression, while the combined treatments with 250 μM FM have shown a statistically significant increase (***, $p<0.001$) of *p53* gene expression as compared to the control, as well as to the individual treatment with 250 μM FM ($p<0.01$) or protons ($p<0.01$) (Figure 1B).

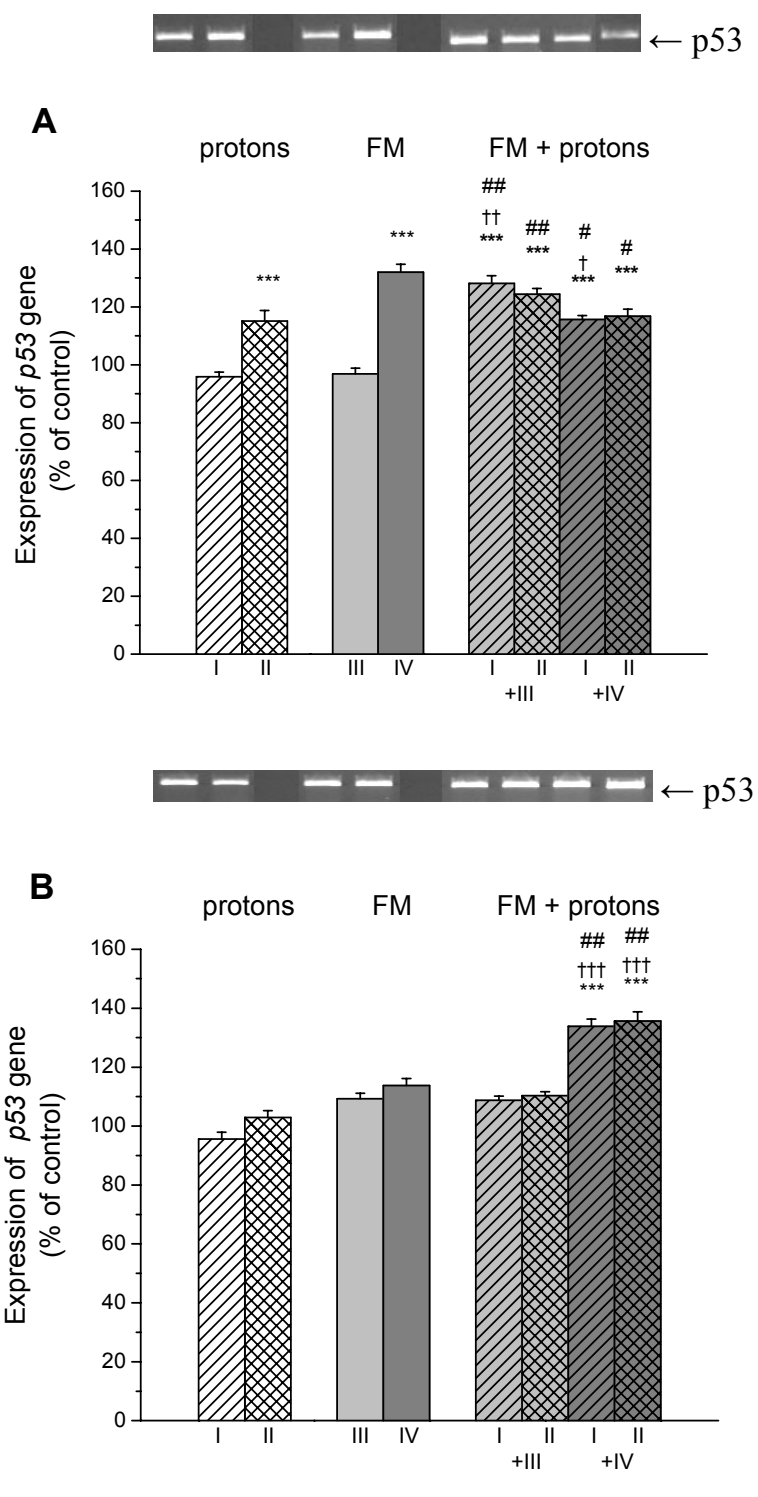

Figure 1: The level of p53 gene expression, 6 h (A) and 48 h (B) after single and combined treatment with FM and protons, expressed in relation to GAPDH. Applied irradiation doses were 12 Gy (I) and 16 Gy (II), while drug concentrations were 100 μM (III) and 250 μM (IV). (* - statistical significance compared to the control; † - statistical significance in relation to the radiation; # - statistical significance compared to the cytostatics; *, †, # - $0.01 < p < 0.05$; **, ††, ## - $0.001 < p < 0.01$; ***, †††, ### - $p < 0.001$).

The effects of DTIC and protons on induction of apoptosis

Percentage of apoptotic cells 6 h after treatment with DTIC did not show significant changes when compared with untreated controls. It was 3.15 and 2.76 % for 100μM and 250 μM DTIC respectively, and 6 to 14 % for combined treatments. Apoptotic index was in the range from 0.77 to 3.91 (Table 1).

Prolonged incubation after treatment with DTIC to 48 h caused similar percentage of apoptotic cells for both concentrations (7.3 - 7.6 %). In combined treatments with protons the percentage of apoptotic nuclei was 3 to 4 % for 100 μM DTIC and 7 % for 250 μM DTIC (Table 1).

The analyses of *p53* gene expression 6 and 48 h after irradiation have shown that single and combined treatments with DTIC and protons did not cause significant changes as compared to the controls (Figure 2B).

DISCUSSION

Melanoma is highly aggressive tumour with a very large metastatic potential. Metastatic melanomas show a lack of sensitivity to current therapeutic approaches. The efficiency of therapeutic agents in the elimination of melanoma is the result of their ability to induce apoptosis in this type of tumour. The use of apoptosis as a therapeutic tool is very important in the case when cell death can be induced by factors with high target specificity. This specificity may be molecular, as in the case of antibodies against tumour-specific antigens, or spatial, if the irradiation is performed using particles characterized by high accuracy of targeting tumours, such as protons [9].

Proton irradiation induced an increase in the number of apoptotic nuclei in HTB140 cells after 6 and 48 h. These results are in agreement with literature data showing that protons induced apoptosis in a similar percentage in other cell types, most of them being radioresistant [2, 9].

FM showed weaker proapoptotic activity than proton irradiation in both analysed time points.

Combined treatments have shown time-dependent effect. The results showed that in the samples exposed to combined treatments the level of apoptosis depends on the concentration of FM, while the increase of radiation dose did not significantly influence the incidence of apoptotic cell death.

The level of expression of the proapoptotic *p53* gene increased in samples exposed to higher FM concentration or higher dose of protons, as well as in all combinations of these two agents 6 h after treatment. 48 h after treatment, the increased level of *p53* is detected in samples exposed to 250 μM FM and protons (12, 16 Gy). The results are in agreement with the data showing that proton radiation led to an increase in the level of mRNA for *p53* in other cell lines [9]. On the basis of these results it can be assumed that the increase in *p53* gene expression is followed by the apoptotic cell death.

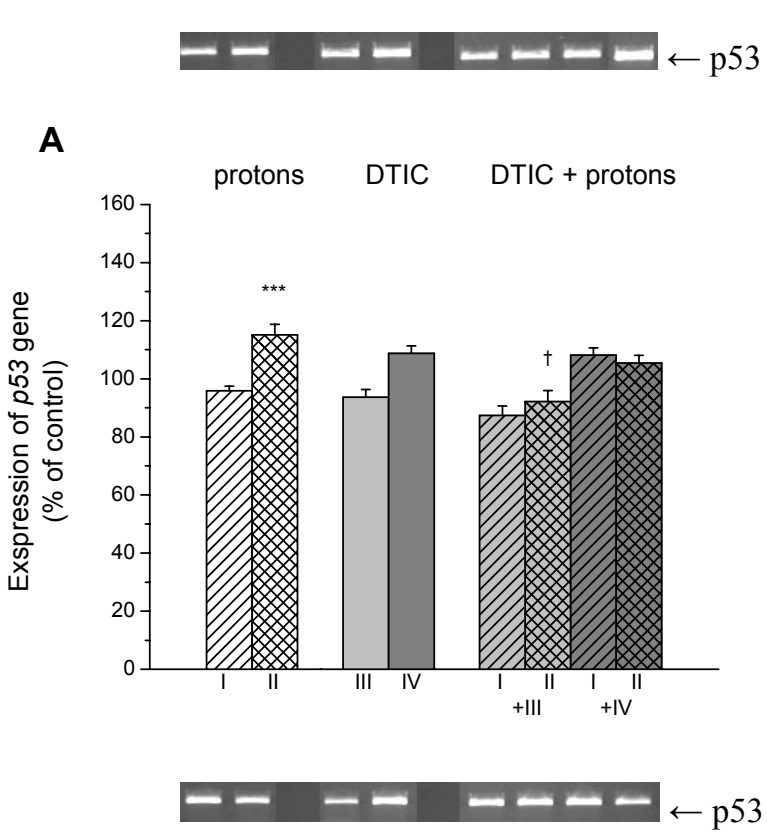

Figure 2: The level of p53 gene expression 6 h (A) and 48h (B) after single and combined treatment with DTIC and protons, expressed in relation to GAPDH. Applied irradiation doses were 12 (I) and 16 Gy (II), while drug concentrations were 100 (III) and 250 μM (IV). (* - statistical significance compared to the control; † - statistical significance in relation to the radiation; # - statistical significance compared to the cytostatics; *, †, # -0.01 <p < 0.05; **, ††, ## - 0.001 <p < 0.01; ***, †††, ### - p < 0.001).

It is known that in radioresistant cells, such as human gastric epithelial tumour cells, radiation induces late apoptosis, that starts 12 h after irradiation, i.e. after release from G_2 arrest and reach maximum values between 72 and 96 h after irradiation [10]. On the basis of these data it can be assumed that combined treatments in our study induce late apoptosis in HTB140 melanoma cells, which is expected due to their high radioresistance [2].

The percentage of apoptotic cells 6 h after single treatment with DTIC was at the level of the control cells, while the best combined effects were with the higher concentration of DTIC and 12 Gy protons. The number of apoptotic cells at 48 h did not show an increase nither after single nor after combined treatments with DTIC and protons.

The lack of induction of apoptosis is in correlation with the fact that DTIC treatment, individually and in combination with protons, did not induce any change in the *p53* gene expression for both analyzed time points.

Considering the fact that DTIC can induce apoptotic cell death in some melanoma cell line [11], the reason that HTB 140 cells do not undergo apoptosis after DTIC treatments could be partly attributed to the specific nature of these cells.

CONCLUSION

The results obtained from this study indicate that combined treatments with FM and protons led to the induction of apoptosis in HTB 140 cells which is related to the increase of *p53* gene expression. On the other hand, combined treatments with DTIC and protons did not induce apoptosis and the level of *p53* gene expression remained unchanged.

ACKNOLEDGMENTS

This work was supported by Ministry of Science and Technological Development of Serbia (grants 143044 and 143038) and INFN-LNS, Italy.

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