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Preliminary study of novel SRC tyrosine kinase inhibitor and proton therapy combined effect on glioblastoma multiforme cell line: *In vitro* evaluation of target therapy for the enhancement of protons effectiveness

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Summary. — The aim of this work was to evaluate proton therapy effectiveness in combination with a molecule SRC protein inhibitor for glioblastoma multiforme treatment. The role of this novel compound, Si306, is to interfere with glioblastoma carcinogenesis and progression, creating a radiosensitivity condition. The experiments were performed on U87 human glioblastoma multiforme cell line. Molecule concentrations of 10 μM and 20 μM were tested in combination with proton irradiation doses of 2, 4, 10 and 21 Gy. Cell survival evaluation was performed by clonogenic assay. The results showed that Si306 increases the efficacy of proton therapy reducing the surviving cells fraction significantly compared to treatment with protons only. These studies will support the preclinical phase realization, in order to evaluate proton therapy effects and molecularly targeted drug combined treatments.

1. – Introduction

Glioblastoma multiforme (GBM) belongs to the group of diffuse astrocytic and oligodendroglial tumor of the gliomas family. The highest grade (IV grade) is assigned to

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GBM, according to the World Health Organization (WHO) classification based on malignancy histological criteria, proliferation index, aggressiveness, response to therapy and life expectancy [1].

The current standard treatment establishes conventional radiotherapy (RT) of 2 Gy for 30 fractions with the alkylating agent temozolomide (TMZ) both concomitantly and RT adjuvant [2]. At present, these treatments are not curative and the median overall survival (OS) is only 14–15 months after diagnosis [3]. Furthermore, radionecrosis and neurocognitive dysfunctions are the main causes of late tissue toxicities of the surrounding organs [4]. Proton therapy (PT), unlike conventional RT, shows physical characteristics which contribute to an overall improved risk-benefit profile in radiotherapy. The reverse depth dose profile of protons allows to hit the cancerous target sparing healthy tissues [5]. For this reason, PT can avoid side effects and increase median OS by means of protocols with dose escalation such as hyperfractionated treatments [6]. Actually, although the demonstration of an overall improved risk-benefit profile and an extension of the OS emerging from clinical trials, some aspects still need to be clarified. In particular, the excessive radiation necrosis and radioresistance phenomena are key features of GBM under investigation [7]. To date, the cellular pathways involved in radioresistance are not fully known. SRC protein non-receptor kinase is one of the main molecular targets involved in GBM radioresistance. In fact, SRC is a key factor which contributes to regulate the main hallmarks of GBM, such as cell morphology, adhesion, migration, invasion, proliferation, differentiation and cell survival [8]. For this reason, the SRC inhibitor compound Si306, has been designed to block the SRC protein activity, with the aim to enhance PT effectiveness and to reduce radioresistance. Computational and modelling analysis have revealed that Si306, can specifically bind the ATP site of the SRC protein making it inactive [9]. In particular, in previous studies it has been demonstrated that Si306 determines a significant reduction in glioblastoma cell proliferation, migration and an enhancement in growth inhibition. Antiproliferative effect of Si306, has been tested in association with X-ray both *in vitro* and *in vivo*. It has been observed that the combination effect of Si306, and RT reduced significantly colony numbers *in vitro* in low-density growth assay compared to the cells treated with only RT. For the *in vivo* studies the combination treatment determined a significant reduction of the tumour growth compared to untreated group [10]. The aim of this preliminary study was to evaluate PT effects in combination with the compound Si306. In our study we tested two concentrations of Si306, 10 μM and 20 μM, combined with PT delivering four doses, 2, 4, 10 and 21 Gy, on U87 human glioblastoma cell line. Our results show an enhancement effect on cell killing by Si306 with proton beam.

2. – Materials and methods

2˙1. *Cell culture*. – The U87 MG human glioblastoma cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cell line was cultured according to ATCC, in Basal Medium Eagle (BME) supplemented with 10% FBS, 1% penicillin/streptomycin, 1% glutamine, 1% non-essential amino acids and sodium pyruvate. Cells were maintained in an exponentially growing culture condition in incubator at 37 °C in a humidified atmosphere (95% air and 5% CO_2) and were routinely sub cultured in 25 cm^2 (T25) standard tissue culture flasks.

2˙2. *Si306 treatment*. – The compound Si306 was kindly provided by Lead Discovery Siena (Siena, Italy). It was dissolved in Dimethylsulfoxid (DMSO, Sigma-Aldrich) with

final concentrations not exceeding 0.5% of DMSO. According to IC_{50} (drug concentration that determined the 50% of growth inhibition) previously calculated [10], U87 cells were pretreated with Si306 concentrations of 10 μM and 20 μM for 24 h. After incubation time, the medium was removed, cells were rinsed two times with phosphate buffered saline (PBS) and fresh medium was added before the irradiations with a proton beam.

2˙3. *Proton irradiation.* – The proton beam irradiation was performed at the CATANA (Centro di Adroterapia ed Applicazioni Nucleari Avanzate) facility of INFN-LNS (Catania, Italy) [11]. It is the first Italian proton therapy facility and it has been in operation since 2002. Here, using 62 MeV of proton beams accelerated by a cyclotron superconducting, patients affected by ocular melanoma are treated. The beamline is composed of several passive elements optimized for the clinical application: scattering foils to spread the beam laterally, collimators to define the beam profile in accordance to the tumor shape and monitor chambers to measure the dose delivered. In order to irradiate the entire T25 flask, a motorized system for biological samples irradiation was used. Radiochromic film detectors were adopted to check lateral dose distribution before each irradiation. The dosimetric system was calibrated under reference conditions according to the International Atomic Energy Agency Technical Reports Series No. 398 "Absorbed Dose Determination in External Beam Radiotherapy" [12, 13]. For combined treatments with 10 μM and 20 μM of Si306, U87 cell line irradiations were carried out using four dose values of 2, 4, 10 and 21 Gy. The same irradiation treatments were performed without the compound Si306, including also dose values of 1, 3 and 6 Gy in order to obtain a clonogenic survival curve as control. Cell irradiations were conducted placing the cell at the middle spread-out Bragg peak, to simulate a clinical condition, with a dose rate of 15 Gy/min.

2˙4. *Clonogenic assay.* – Two days before treatments, U87 cells were seeded in T25 flasks at a density of 3×10^5/flask and maintained at subconfluence. After irradiation, the cell survival was performed by clonogenic assay according to the protocol of Franken *et al.* [14] Briefly, after irradiation, U87 cells were detached, counted by haemocytometer and seeded in a 6-well plate in triplicate at a density of 50–2000 cells per well according to the dose delivered to assay the surviving fraction (SF). The number of cells plated was chosen to yield at least 50 colonies per flask. After an incubation time of 12 or 14 days, cells were fixed with 50% methanol for 20 min and stained with 0.5% crystal violet (both from Sigma-Aldrich, St. Louis, MO, USA). Colonies with more than 50 cells were counted as clonogenic and SF determined according to the plating efficiency (PE) of untreated cells (control).

3. – Results

The effect of Si306 alone and in combination with PT was assessed in U87 cells. After cell exposure with Si306 alone at concentration of 10 μM and 20 μM, we observed a SF of 80% and 60%, respectively, showing a dose-response relationship. Following proton irradiation with doses of 2, 4, 10 and 21 Gy, the SF of the U87 cells without the drug obtained were as follows: 40%, 21%, 7% and 3%. Cell survival was further reduced after the pre-treatment with Si306 combined with the irradiation treatment. SF obtained after combined treatments were as follows at the same irradiation doses: 28%, 20%, 5% and 3% for the setting with 10 μM of Si306; 16%; 10%, 4% and 2% for the setting with 20 μM of Si306. The results of clonogenic assays for U87 cell lines after irradiation with protons alone and in combination with Si306 are shown in fig. 1.

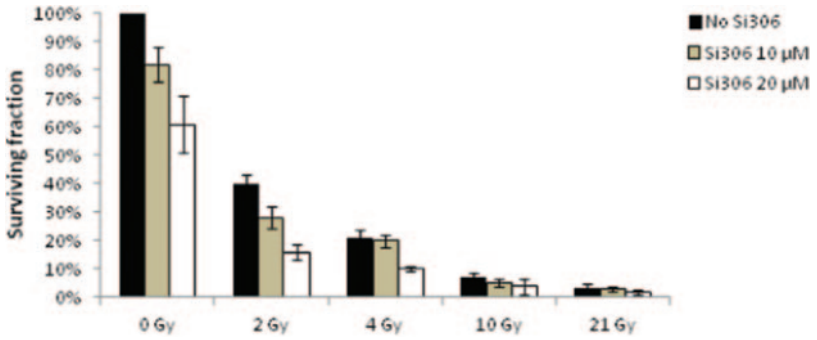

Fig. 1. – Effect of Si306 in combination with proton therapy on the human U87 glioblastoma cell line. Bar diagrams of surviving fraction are in percentage. The data are mean ± SD of three independent experiments.

4. – Discussion

In this study, we evaluated the effect of PT combined with a novel molecule inhibitor of SRC on the glioblastoma cell line using clonogenic assay. The work shows that pretreatment with the compound Si306 contribute to weak clonogenic activity of glioblastoma cells irradiated with proton beams. In addition to the radiosensitivity evaluation, one of our main goals is to investigate IR-induced radioresistance at molecular level since gene expression profiling may reflect different clinical outcomes by assuming a significant prognostic value of gene signatures to predict a glioblastoma response to the radiation treatment. Therefore, gene expression analyses by whole-genome cDNA microarray are in progress in order to evaluate ionizing radiation-induced pathways that can be modulated by Si306 activity. There are no available studies about gene signatures proton-induced, especially in combination with targeted molecules.

From a clinical perspective, PT might be a promising treatment for patients with GBM and the inhibition of SRC tyrosine kinase proteins is a favourable strategy to overcome invasion, migration and other mechanisms involved in radioresistance of GBM. Previous studies have shown that inhibition of SRC proteins reduces the expression of vascular endothelial growth factor (VEGF) and invasive processes that can be triggered by direct inhibitors of VEGF, such as bevacizumab or by exposure to IR itself [15-17]. Over recent years, few *in vitro* studies have been performed about the proton effects in association with molecular targeted drugs for GBM treatment. Among the studies with particle therapies, most of the information is related to the evaluation of the effect of high-LET particles, such as carbon ions combined with TMZ or other chemotherapeutic agents [18-20]. Although a greater radiobiological efficacy of carbon ions has been shown for glioblastoma cell lines compared to photon irradiation, we encourage to implement studies that guide towards a new clinical trial for PT. One of the main reasons is the limited availability of dedicated facilities for carbon ion therapy compared to PT centres: more than 100,000 patients have been already treated in 50 centres for cancer treatment with protons. Carbon ion centres instead are located in few countries, since only two of them are in Europe: Heidelberg Ion-Beam Therapy Centre (HIT) in Heidelberg, Germany and Centro Nazionale di Adroterapia Oncologica (CNAO) in Pavia, Italy [21, 22].

This work shows for the first time the effects following the combination of proton irradiation with a molecular targeted agent blocking SRC protein in the GBM cell line.

Therefore, our *in vitro* results represent radiobiological data useful for subsequent preclinical steps, as well as clinical applications, contributing to define a personalized biologically driven treatment plan.

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