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Proton and photon beams interaction with radiosensitizing agents in human glioblastoma cells

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Summary. — In oncological field, chemoradiotherapy treatments that combine radiations to radiosensitizing chemical agents are spreading out. The aim of this kind of treatment is to obtain a better tumor local control and at the same time to reduce the distant failure. The combination of radiation with microtubule-stabilizing agents is very promising in cancer therapy. In the present study, the combination of clinical proton beams and the microtubule-stabilizing agent Epothilone B has been investigated in human glioblastoma cells cultured *in vitro*. Photon beams have been used for comparison. Cell survival has been evaluated by colony forming assay and the interaction mechanism between radiation and Epothilone B has been investigated: survival curves relative to the combined treatment (protons or photons with Epothilone B) showed a linear trend, different from the linear quadratic behavior found with radiation alone. The analysis performed showed a synergism in the radiation-drug interaction. Thus, Epothilone B in conjunction with radiation acts as a radiosensitizer. Finally proton Relative Biological Effectiveness has been determined and results are reported in this paper.

1. – Introduction

In recent years, cancer treatment modalities that combine the use of radiation (photons or charged particles) to chemotherapy agents are spreading out. The main goal of this new kind of therapy is to achieve a better tumor local control and at the same time reduce the risk of distant metastasis.

Nowadays, an increasing number of studies are investigating the interaction of chemotherapy not only with photons, but even with charged particles for the treatment of aggressive and resistant tumors [1-3], associated to a very poor prognosis, such as glioblastomas (GM). GM are highly invasive primary tumors of the central nervous system. Such an invasive behaviour is one of the main obstacles in the treatment of this cancer. The benefits of the use of charged-particle therapy instead of photon conventional radiotherapy in the treatment of this type of tumors are due to dosimetrical and

radiobiological properties. In particular, the main advantage of protons with respect to photons is their dose spatial distribution, characterized by the Bragg Peak at the end of particle tracks. This permits to escalate the dose to the tumor, sparing the surrounding normal tissues.

The research of drugs to be used in conjunction with radiation in oncological treatments has recently led to the discovery of the Epothilones, a new class of microtubule-stabilizing agents (MSAs). MSAs are able to interfere with the mitotic spindle formation [4], leading to cell cycle arrest in the G2/M phase, that is the most radiosensitive phase in the cell cycle [5,6]. For this reason, MSAs can be used not only as chemotherapy drugs because of their cytotoxicity, but also as radiosensitizing agents. When compared with other MSAs, such as the Taxanes, the Epothilones result effective at lower concentration and their water solubility avoids the use of other excipients, thus inducing fewer side effects [7].

In the present study, we have investigated the interaction of clinical proton beams (and, for comparison, photon beams) with Epothilone B (Patupilone, EPO906), a chemical agent that has been studied both *in vitro* and *in vivo* even in conjunction with photon beams [8] [9]. In clinical trials it has been used as a chemotherapy drug even for the treatment of glioblastomas [10], and recently it has also been combined with photon radiotherapy for the treatment of brain malignancies, with encouraging results [11].

Presently in literature there are no published data about proton irradiation combined with Epothilone B. Thus, the purpose of this study is to evaluate *in vitro* the effects of proton beams combined to Epothilone B in glioblastoma multiforme U251MG cells in term of clonogenic survival. Cell clonogenic survival has been evaluated after irradiation alone or combined with Epothilone B. Results have been compared with the results of analogous measurements with photon clinical beams and finally proton Relative Biological Effectiveness (RBE) has been evaluated. Despite the fact that proton therapy is nowadays widely used for some type of cancer, there are still a lot of aspects to be clarified in the biological response of cells irradiated with protons [12], among which the RBE.

2. – Material and Methods

2˙1. *Cell cultures*. – U251MG cells were maintained as exponentially growing cultures at 37 °C in humidified atmosphere containing 5% CO2 in air in Eagles Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS) and gentamicin (50 M/ml). In these conditions the doubling time was estimated to be 24 ± 1 hours.

2˙2. *Drug preparation and cell treatment*. – Epothilone B was dissolved in dimethyl sulfoxide (DMSO) to generate a 10 M stock solution that was diluted in medium at appropriate concentrations.

In order to quantify the drug concentration to be used in conjunction with radiation, clonogenic survival was measured after 24-hour treatment with Epothilone B at concentrations up to 0.6 nM. A concentration of 0.125 nM, corresponding to the 40% of clonogenic survival, was chosen to be used in conjunction with irradiation.

24 hours before irradiation, half of the cells was treated with Epothilone B 0.125 nM. The drug was removed just before irradiation and the flasks with cells undergoing irradiation were completely filled with the medium.

2˙3. *Irradiation.* – Protons irradiation was performed with the synchrotron-based clinical scanning beams at the Centro Nazionale di Adroterapia Oncologica (CNAO, PAVIA) [13, 14]. The flasks were placed inside a motorized water phantom in a uniformly scanned $10 \times 10\,\mathrm{cm}^2$ field size. They were put at the isocenter, in the mid Spread-Out Bragg Peak (SOBP)(15 cm depth). The SOBP (from 12 to 18 cm depth) was obtained with active beam energy modulation, using 16 different energies between 131.5 and 164.8 MeV. Protons Linear Energy Transfer (LET) in this position, evaluated with Monte Carlo FLUKA simulation, was $3.6\,\mathrm{keV}/\mu\mathrm{m}$.

The samples were irradiated at different doses between 0 and 5 Gy.

Photon beam irradiation was performed with a 6 MV linear accelerator at the Fondazione IRCCS Istituto nazionale dei Tumori, Milano. The flasks were placed in the center of a uniformly irradiated $20 \times 20\,\mathrm{cm}^2$ field at the isocenter (5 cm depth, vertical beam).

The samples were irradiated at different doses between 0 and 7 Gy.

At least three independent experiments have been done for both protons and photons.

2˙4. *Clonogenic Survival Assay.* – After being irradiated, cells (pre-treated or not with Epothilone B) were detached using 0.25% Trypsin-EDTA. Then they were counted, seeded in 5 T25 flasks for each dose and incubated for 13 days. After this time they were fixed with ethanol and stained with 10% Giemsa solution.

Colonies made up of more than 50 cells were counted as survivors.

2˙5. *Analysis of radiation-drug interaction.* – Dose-survival curves relative to the treatment with radiation alone or in conjunction with Epothilone B were analyzed in order to evaluate if the interaction mechanism between radiation and drug was simply additive or synergistic.

In order to perform this analysis, we applied a method proposed by Luttjeboer *et al.* [15]: in the dose-effect plane an additivity region is identified between two survival curves calculated for two different additivity mechanisms. In the "independent" additivity mechanism, drug and radiation are supposed to act independently, while in the "overlapping" one, the drug is assimilated to an additional radiation dose. Experimental curves relative to the combined use of radiation and drug that fall below this region indicate a synergism in the radiation-drug interaction. Otherwise, experimental curves falling inside this region indicate a simply additive interaction.

3. – Results

3˙1. *Radiation-Drug interaction.* – Figure 1 shows the surviving fractions (S.F.) of cells irradiated with photon beams alone or in conjunction with Epothilone B. The dashed and dotted lines represent the "independent" and "overlapping" additivity curves calculated according to the method suggested by Luttjeboer *et al.* [15].

Solide curves are the fit of the experimental data according to the Linear Quadratic model. Data relative to irradiation alone have been fitted with the function

$$\mathrm{S.F.} = e^{-\alpha D - \beta D^2},$$

where D is the radiation dose.

The fit of the experimental data relative to the combination of radiation and Epothilone B, shows that the quadratic term becomes consistent with 0, thus these

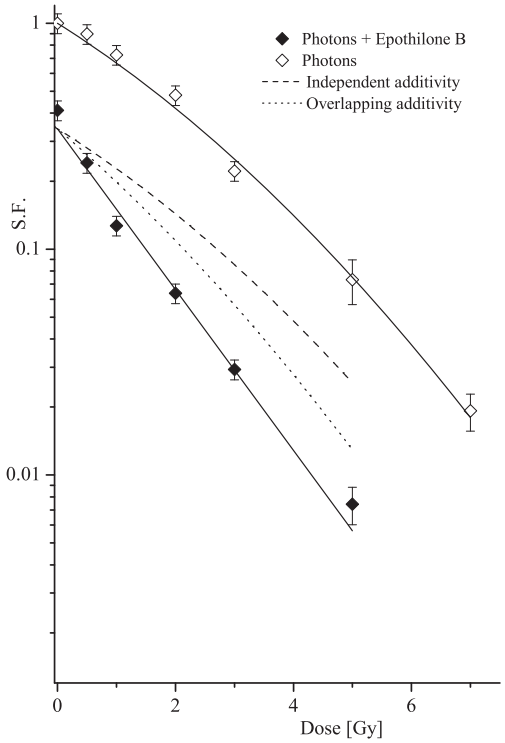

Fig. 1. – Surviving fraction (mean of at least 3 independent experiments) of cells exposed to photon beams alone or in conjunction with Epothilone B. Error bars are the maximum value between the 10% of survival and the mean standard error. The dashed and the dotted curves represent the "independent" and "overlapping" additivity respectively. Reported data are a part of a study already submitted for publication [16].

data have been fitted with the function

$$\text{S.F.} = S_0 e^{-\alpha D},$$

where S_0 is the surviving fraction of cells treated with Epothilone B and not irradiated. This fact indicates that Epothilone B modifies cell response to irradiation, reducing the shoulder typical of survival curves obtained after low-LET (Linear Energy Transfer) irradiation.

The curve relative to the combined treatment is located below the additivity region bounded by the independent and overlapping additivity curves, thus indicating a synergism in radiation-drug interaction.

Analogous considerations can be done looking at the survival curves relative to proton irradiation with and without Epothilone B, reported in fig. 2. Also in this case, in the fit of experimental data relative to irradiation combined with Epothilone B, the quadratic term becomes negligible and the curve falls below the additivity region, indicating a synergism in radiation-drug interaction. This synergism seems to be slightly weaker than what observed with photons.

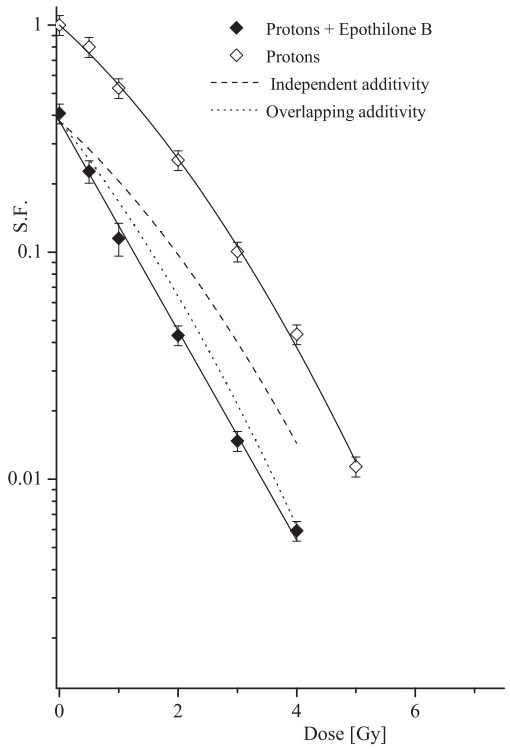

Fig. 2. – Surviving fraction (mean of at least 3 independent experiments) of cells exposed to proton beams alone or in conjunction with Epothilone B. Error bars are the maximum value between the 10% of survival and the mean standard error. The dotted and the dashed curves represent the "independent" and "overlapping" additivity respectively. Reported data are a part of a study already submitted for publication [16].

3˙2. *Relative Biological Effectiveness*. – Protons RBE was calculated as the ratio between the dose of photons and the one of protons necessary to produce the same biological effect (*i.e.* the same survival level). At 10% of survival, the RBE of the CNAO therapeutic proton beams relative to 6 MV photons in the mid SOBP for U251MG cells resulted 1.4 ± 0.1; this value is greater than the clinically assumed one for proton RBE, equal to 1.1–1.2.

4. – Discussion

In the present study, the clonogenic survival of U251MG glioblastoma cells has been investigated after protons or photons irradiation alone or in conjunction with Epothilone B.

For both protons and photons, the dose-survival curve after the treatment with radiation alone showed a linear-quadratic behaviour. The use of Epothilone B modifies cells response to irradiation, removing the shoulder of dose-survival curves.

The analysis performed to investigate the interaction modality between radiation and Epothilone B, showed a synergism in radiation-drug interaction.

In literature there are some published *in vitro* studies investigating the interaction between photon beams and Epothilone B. Baumgart *et al.* [17] found that Epothilone B interaction with photon beams is synergistic in human epithelial cancer cells. Hofstetter *et al.* [8] found that Epothilone B has a radiosenziting effect on human colon adenocarcinoma cell line SW480 and in p53-null MEF cells when used in conjunction with X-rays. Bley *et al.* [18] found an at least additive effect of Epothilone B and X-rays on A549 cell line.

To the author knowledge, there are no published studies on the use of Epothilone B in conjunction with proton beam irradiation on U251MG cells. The present study shows that Epothilone B increases protons toxicity and that the synergism in radiation-drug interaction is slightly weaker for protons than for photons. This may be due to a different effectiveness of these two types of radiation. In fact, proton RBE at 10% in the mid SOBP resulted 1.4 ± 0.1. This value is higher than the clinical assumed one (1.1–1.2) for therapeutic proton beams [19]. As a matter of fact, *in vitro* values of proton RBE published in the literature show significant variations as reported in a review by Paganetti [20]. Moreover, recent reviews suggest that cells biological response is differentially modulated by protons and photons for several end points [12, 21]. Thus, proton RBE still needs to be further investigated.

In conclusion, this study shows that Epothilone B in combination with proton or photon beams might act as a radiosensitizer, with a synergistic modality of interaction with radiation. These promising results could be a basis for further experiments and for clinical studies.

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