

Combining proton or photon irradiation with Etoposide B. An *in vitro* study of cytotoxicity in human cancer cells.

Daniela Bettega^{(1) (2)}, Paola Calzolari^{(1) (2)}, Mario Ciocca⁽³⁾, Angelica Facchetti⁽³⁾, Miriam Lafiandra^{(1) (2)}, Renato Marchesini⁽¹⁾, Silvia Molinelli⁽³⁾, Emanuele Pignoli⁽⁴⁾, Barbara Vischioni⁽³⁾

⁽¹⁾ Dipartimento di Fisica, Università degli Studi di Milano

⁽²⁾ Istituto Nazionale di Fisica Nucleare (INFN), Milano, Italy

⁽³⁾ Centro Nazionale di Adroterapia Oncologica, Pavia, Italy

⁽⁴⁾ Fondazione IRCCS Istituto Nazionale dei Tumori, Milano, Italy

Corresponding author: Daniela Bettega, Dipartimento di Fisica, Via Celoria 16-20133 Milano, Italy Tel: ++390250317210; Fax: ++390250317208; email: daniela.bettega@mi.infn.it

Abstract:

Recently, the use of proton beams in cancer therapy is spreading out and tumour treatment modalities combining radiosensitizing chemical agents with irradiation are under investigation in order to achieve greater tumour local control and reduce the probability of distant failures. The combined treatment modality of radiation and the clinically relevant microtubule-stabilizing compound Etoposide is a promising approach for anticancer therapy. In the present study, we investigated the cytotoxicity of a Spread Out Bragg Peak (SOBP) proton beam, as well as of 6 MV photons, in human glioblastoma (U251 MG) and lung adenocarcinoma (A549) cells pretreated for 24 h, or not, with Etoposide at concentrations of 0.125 and 0.075 nM respectively. Proton irradiation was performed at the middle position of an actively modulated SOBP (12 -18 cm depth in water) and cell survival was evaluated by colony forming assay.

For both cell lines, survival curves after proton or photon irradiation alone showed a linear quadratic behaviour with a proton RBE (Relative Biological Effectiveness), compared with photons at 10% survival, of 1.5 ± 0.2 . Treatment of the cells with Etoposide at subnanomolar concentration has an antiproliferative effect. Furthermore, differently from the results found with radiation alone, the survival curves for the combined treatment Etoposide - radiation showed a linear trend and analysis of the interaction of the two cytotoxic agents indicated a slight synergism.

These data provide a radiobiological basis for further experiments, as well as clinical studies.

1. Introduction

Lung cancer remains the most common cause of cancer death in western countries (Jemal *et al* 2009, Ferlay *et al* 2008). Lung adenocarcinoma is a kind of non-small cell lung cancer (NSCLC) that affects the epithelial cells of the bronchial tube and it is the most common lung neoplasia in non-smokers. Recently, new treatment modalities, such as concurrent chemoradiotherapy (O' Rourke *et al* 2010, Eberhardt 2015, Bao *et al* 2015) and particle radiotherapy (Liao *et al* 2011, Grutters *et al* 2010), have been introduced for patients with NSCLC. The rationale for combining chemotherapy and radiotherapy is to exploit the benefits of radiotherapy in terms of sensitising tumour to radiation locally and reducing the risk of distant failures. Many studies have evaluated the combined effect of radiation and chemotherapy using photons. Combined radio-chemotherapy protocols are already used in the treatment of many cancers, such as glioblastoma multiforme, pancreas cancer or non-small-cell lung cancer (Durante 2014, Albain *et al* 2009).

Glioblastomas (GBMs) are the most frequent primary tumours of the central nervous system. They are very aggressive, highly angiogenic and typically associated with very poor prognosis. One of the main biological features of GBM is the local invasion of the surrounding brain tissue. Such an invasive behaviour represents the greatest obstacle to an effective treatment of this kind of brain tumour (Pagano *et al* 2012). Currently, the standard approach to glioblastoma treatment combines surgical resection, chemotherapy and radiotherapy. Beyond initial surgery aimed at reducing the tumour burden, the mainstay of therapy is based on the use of concurrent and adjuvant Temozolomide (TMZ, a DNA-binding agent) in conjunction with radiotherapy (Stupp *et al* 2009, Sathornsumetee and Rich 2008, Mirmanoff *et al* 2006, Stupp *et al* 2015).

An increasing number of studies are investigating the interaction of drugs with charged particles, namely carbon ions and protons, which are very promising for the treatment of radioresistant tumours due to their dosimetric and radiobiological properties (Combs *et al* 2012, Kitabayashi *et al* 2006, El Shafie *et al* 2013, Schlaich *et al* 2013, Loeffler and Durante 2013, Chalmers *et al* 2009, Barazzuol *et al* 2012). For protons, the major difference with photon radiotherapy is the spatial distribution of the absorbed dose: using charged particles for the treatment of tumour close to vital and radiosensitive organs, the dose deposited to the surrounding normal tissue can be reduced and therefore the dose to the

tumour escalated. Thus, proton beam therapy is suitable when normal tissue sparing is a priority such as in the treatment of lung cancer, given the proximity to the oesophagus, the heart and the spinal cord. The first study on proton therapy with concurrent chemotherapy with carboplatin and paclitaxel for unresectable stage III non-small cell lung cancer was reported by Chang (Chang *et al* 2011) with good results in terms of patients median survival and toxicity. As far as we know, very few data are available on the effect of proton irradiation alone or in combination with chemotherapy in patients with GBM (Combs *et al* 2010, Rieken *et al* 2012, Fitzek *et al* 1999, Suit *et al* 2008).

The research of drugs to be used as radiosensitisers has led to the discovery of a new class of Microtubule-stabilizing agents (MSAs): the Epothilones. MSAs, such as the widespread Taxanes, are able to interfere with the mitotic spindle formation, leading to cell cycle arrest in the G2/M phase, the most radiosensitive phase in the cell cycle (Bollag *et al* 1995, Altmann *et al* 2000, Altmann and Gertsh 2007, Agrawal *et al* 2003, Pawlik and Keyomarsi 2004, Roher Bley *et al* 2013). Epothilone B (Patupilone, EPO906) has been used in several clinical trials (Vansteenkiste *et al* 2007, Oehler *et al* 2012) and has been tested as a chemotherapy drug both *in vitro* and *in vivo*, even in combination with photon radiotherapy (Hofstetter *et al* 2005, Kim *et al* 2005). A recent phase I trial investigated Epothilone B in conjunction with irradiation in patients with recurrent glioma (Fogh *et al* 2010). The ability of Epothilone B to cross the blood-brain barrier and to retain in brain tissue (O'Reilly *et al* 2008) makes it a very powerful and promising chemotherapeutic agent for brain malignancies. Epothilone B has shown antivasular and antiangiogenic effects (Ferretti *et al* 2005, Bocci *et al* 2002) and the ability to effectively inhibit cells' migration at non-cytotoxic concentration (Pagano *et al* 2012, Furmanova-Hollenstein *et al* 2013). It was also demonstrated that Epothilone B reduces DNA repair capability of tumour cells (Baumgart *et al* 2012, 2015) .

The purpose of this study was to investigate the effect of Epothilone B combined with proton or photon beams on cultured human tumour lung cells, A549, and glioblastoma multiforme cells, U251MG. Cell clonogenic survival was assessed after irradiation alone or combined with Epothilone B, in order to determine the interaction mechanism between radiation and drug.

Protons RBE (Relative Biological Effectiveness) for A549 and U251MG cells clonogenic survival was determined by comparing cell survival data after proton and photon irradiation. Indeed, although proton

therapy is a consolidated alternative to photon radiotherapy for some types of cancer and an RBE of 1.1 has been recommended for clinical use, proton RBE values reported in literature show large fluctuations (see for a review Paganetti 2014, Jones 2016). Furthermore data published in recent years on several responses of cells irradiated with protons and, for comparison, to photons (see for a review Tommasino and Durante 2015), have indicated that RBE in proton therapy is still an issue that needs further investigation.

2. Methods

2.1. Cell lines and culture

A549 and U251MG cultures were supplied by the ICLC (Interlab Cell Line Collection, Genova, Italy). A549 and U251MG cells were maintained at 37°C in humidified atmosphere containing 5% CO₂ in air as exponentially growing cultures respectively in Dulbecco Modified Eagles Medium (DMEM, Sigma-Aldrich) and Eagles Minimum Essential Medium (EMEM, Sigma-Aldrich), both supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and gentamicin (50 µg/ml) (Sigma-Aldrich). In these conditions the doubling time were 21 ± 0.2 hours and 24 ± 1 hours for A549 and U251MG respectively. All cell lines were confirmed to be Mycoplasma-free before use. The cells were plated in T25 flasks (10^5 cells/ flask) 72 h before the irradiation.

2.2. Epothilone B

Epothilone B (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) to generate a 10µM stock solution. The stock solution was then diluted in medium at appropriate concentrations. The final DMSO concentrations were less than 0.1%, a concentration which had no effect on cell survival. For the combined treatments, 24 h before the irradiation Epothilone B was added to the samples using equitoxic concentrations for the two cell lines (40% clonogenic survival, 0.075 nM and 0.125 nM respectively for A549 and U251MG cells, see 3.1). The drug was removed after 24 h, just before the irradiation.

2.3 Irradiation

Cell irradiation with protons was performed using the synchrotron-based clinical scanning beams (fixed horizontal beam line) at the Centro Nazionale di Adroterapia Oncologica (CNAO, PAVIA) (Mirandola *et al.* 2015, Rossi 2015). The flasks were placed vertically inside a water phantom put at the isocenter on the treatment table, at the depth of 15 cm, corresponding to the mid Spread-Out Bragg Peak (SOBP).

The SOBP (6 cm width, from 12 to 18 cm depth in water) was achieved with active beam energy modulation, using 16 different energies (131.5-164.8 MeV). The flasks were put in the centre of an uniformly scanned 10x10 cm² field size (scanning step equal to 3 mm). Protons dose-averaged Linear Energy Transfer (LET) in the mid SOBP, evaluated with Monte Carlo FLUKA simulation, was 3.6 keV/μm. Samples were irradiated at different doses (0-5 Gy).

Photon beam irradiation of cell cultures was performed using a 6 MV linear accelerator (VARIAN Clinac 2100C, Varian Medical Systems, Palo Alto, USA) at the Fondazione IRCCS Istituto Nazionale dei Tumori, Milano. The flasks containing the cells were placed horizontally at the isocentre in a water phantom at 5 cm depth and were irradiated using a vertical beam 20x20 cm² field. Samples were irradiated at different doses (0-7 Gy).

For both the beams all the flasks were irradiated completely filled with medium.

2.4. *Clonogenic Assay.*

The assessment of the effect of the combination of Etoposide B and radiation (photons or protons) was performed using the clonogenic assay. Retention of reproductive integrity, i.e. the ability of a cell to produce a viable colony containing at least 50 cells, is the accepted gold standard for measuring the radiosensitivity of a cell population (IAEA 2010). Furthermore clonogenic survival has a particular relevance to the radiotherapy of tumours as for a tumour to be eradicated it is necessary that the cells are made unable to divide and cause further growth of the malignancy (Hall and Giaccia 2006).

To measure cell clonogenic survival, cells were detached from the flask immediately after irradiation using 0.25% Trypsin-EDTA, counted, reseeded in 5 T25 flasks for each dose/ treatment at suitable concentration and incubated for about 13 days. Incubation time intervals for clonogenic survival of these human cell lines reported in the literature are between 10 and 14 days (Baumgart *et al* 2012, Combs *et al* 2012, Rohrer Bley *et al* 2009). The cells were then fixed with ethanol and stained with 10% Giemsa solution and colonies consisting of more than 50 cells were scored as survivors. Surviving fractions relative to the untreated samples were determined.

2.5. *Analysis of radiation-drug interaction*

Survival curves obtained with radiation alone and in conjunction with Etoposide B were analysed to determine whether the interaction between radiation and drug was additive or synergistic.

According to Luttjeboer (Luttjeboer *et al.* 2010), for two combined treatments, i.e. drug and radiation, it is possible to identify an additivity region in the dose-effect plane, bounded by two survival curves calculated for two different additivity mechanisms, i.e., “independent” and “overlapping”. In the first case it is supposed that drug and radiation act independently, therefore survival after the combined treatment is calculated as the product of the survival values relative to the single treatments. For “overlapping” additivity the drug is supposed to act as an additional radiation dose, D^* . D^* is calculated as the dose giving the same cytotoxicity induced by the drug alone. A survival curve for the combined treatment located below this additivity region suggests the presence of a synergistic radiation-drug interaction.

3. Results

3.1 Epothilone B effects on clonogenic survival

The cells were exposed to Epothilone B concentrations between 0.05 nM and 0.6 nM, typical of a clinically achievable range of drug concentrations (Woltering *et al.* 2003). Fig.1 shows the clonogenic surviving fraction of A549 (circles) and U251MG cells (squares) as a function of Epothilone B concentration. No effect occurred at concentrations lower than 0.05 nM and 0.075 nM for A549 and U251MG respectively thus suggesting the presence of a threshold concentration under which the drug has basically no effect on cell clonogenic survival. For concentrations greater than the threshold value, clonogenic survival decreases. A concentration corresponding to a surviving level of about 40%, was chosen to be used in conjunction with radiation. This concentration is equal to 0.075 nM for A549 cells and to 0.125 nM for U251MG. Results of the present study on A549 cells treated with Epothilone B at concentrations between 0,05 and 0.1 nM are similar to those found for clonogenic survival by Rohrer Bley (Rohrer Bley *et al.* 2009) whereas results on U251MG cells at EpothiloneB concentrations between 0.05 and 0.2nM are similar to those found by Furmanova-Hollenstein (Furmanova-Hollenstein *et al.* 2013).

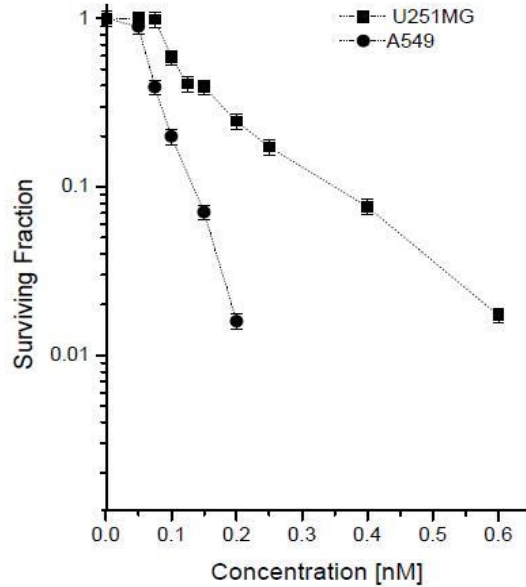


Fig. 1 Surviving fraction of A549 (●) and U251MG (■) cells as a function of Epothilone B concentration. Error bars represent SE (SE = Standard Error).

3.2. Clonogenic survival vs radiation dose and the effect of Epothilone B .

Figures 2 and 3 show survival data (mean of 4 independent experiments) of A549 and U251 cells exposed to (a) 6MV photons and (b) protons, alone (full symbols) or combined with Epothilone B (empty symbols). The curve relative to radiation alone is the fit to the experimental points according to the linear quadratic model, $S = \exp(-\alpha D - \beta D^2)$ (eq.1) with S = Survival and D = Dose.

Data obtained for cells treated with Epothilone B show a different radiation dose-dependence. In fact, the surviving fraction decreases exponentially with dose, without any shoulder. Thus, data were fitted with a linear function $S = S_0 \exp(-\alpha D)$ (eq.2), where the parameter S_0 represents the clonogenic survival of cells treated with Epothilone B and not irradiated. The value obtained for the fit parameters are reported in Table 1.

Comparing the curves relative to photon and proton irradiation alone, it is possible to notice that for both cell lines, protons are more effective than photons in inducing clonogenic cell death. Indeed survival values at 2 Gy (SF2Gy) with A549 cells are 0.49 ± 0.05 and 0.25 ± 0.03 for photons and protons respectively, with U251MG cells SF2Gy results are 0.48 ± 0.05 and 0.25 ± 0.02 for photons and protons respectively. Protons Relative Biological Effectiveness (RBE) was calculated as the ratio between the dose of reference radiation (6MV photons) and that of protons necessary to produce the

same biological effect. At 10% of clonogenic survival the RBE of the CNAO proton beam (mid SOBP) resulted equal to 1.5 ± 0.2 for both the cell lines.

For combined treatments with Etoposide B, protons effectiveness relative to combined treatments with photons can be evaluated as the ratio of the slopes of the two exponential survival curves (α_p/α_x). In this case, it did not depend on the survival level and its value was 1.3 ± 0.1 for both A549 and U251MG cells.

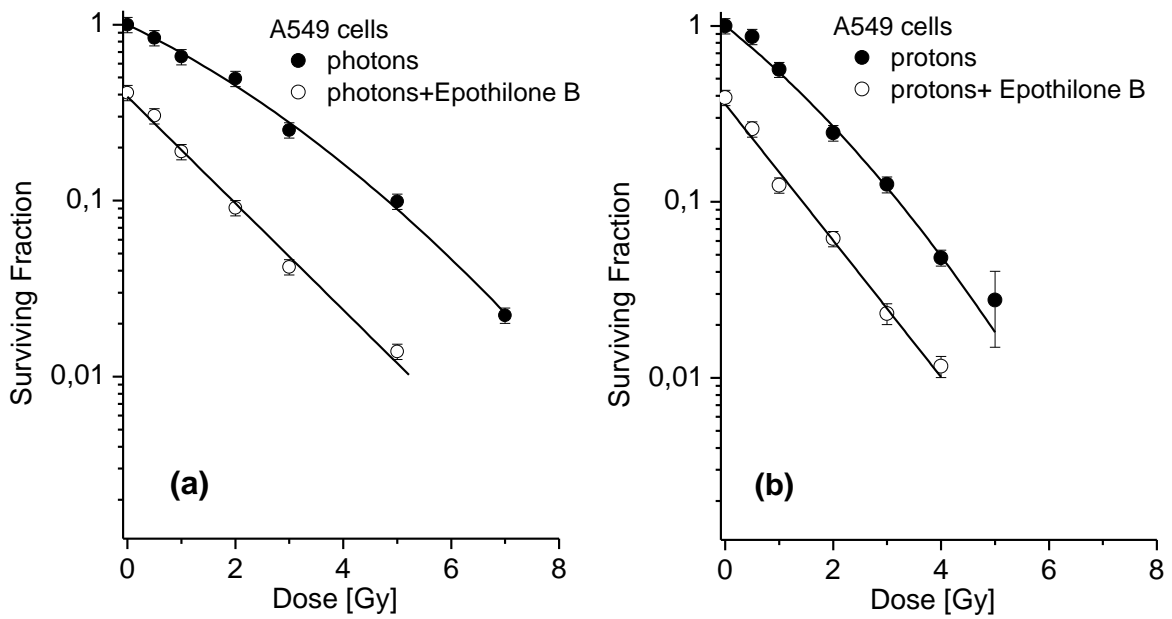


Fig. 2 Surviving fraction (mean of 4 independent experiments) of A549 cells exposed to (a) photon and (b) proton beams alone (●) and in combination with 0.075 nM Epothilone B (○). Solid lines are the fit of the experimental data according to eqs (1) or (2) for radiation alone or combined with Epothilone B respectively. Error bars represent SE (SE = Standard Error)

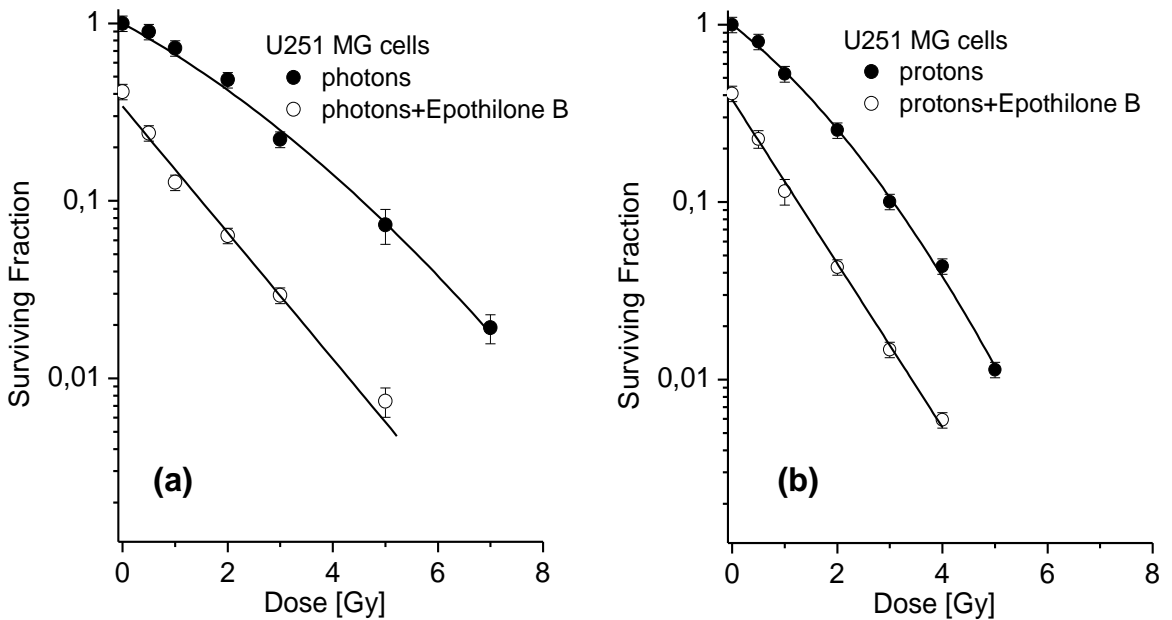


Fig.3 Surviving fraction (mean of 4 independent experiments) of U251MG cells exposed to (a) photon and (b) proton beams alone (●) and in combination with 0.125 nM Epothilone B (○). Solid lines are the fit of the experimental data according to eqs (1) or (2) for radiation alone or combined with Epothilone B respectively. Error bars represent SE (SE = Standard Error)

Table 1: Parameters of Survival Curves

Radiation	Cell line	Radiation alone		Radiation + Etoposilone B	
		$\alpha \pm \text{SE} [\text{Gy}^{-1}]$	$\beta \pm \text{SE} [\text{Gy}^{-2}]$	$S_0 \pm \text{SE}$	$\alpha \pm \text{SE} [\text{Gy}^{-1}]$
photons	A549	0.34 ± 0.04	0.028 ± 0.006	0.39 ± 0.03	0.70 ± 0.04
	U251MG	0.38 ± 0.04	0.028 ± 0.009	0.34 ± 0.02	0.82 ± 0.04
protons	A549	0.56 ± 0.07	0.05 ± 0.02	0.36 ± 0.03	0.89 ± 0.04
	U251MG	0.54 ± 0.05	0.07 ± 0.01	0.38 ± 0.03	1.06 ± 0.03

Notes α and β values were obtained from the fit of experimental data for radiation alone or in combination with Etoposilone B according to eqs (1) or (2) respectively. S_0 is the clonogenic survival of cells treated with Etoposilone B alone. SE= Standard Error.

3.3 Interaction between radiation and Etoposilone B

Figures 4 and 5 show the results of the analysis of radiation–drug interaction for A549 and U251MG cells, respectively. The experimental data relative to the combined treatment (CT) are compared with the curves calculated for independent (IA) and overlapping (OA) additivity of the two agents.

The doses giving the same cytotoxicity as Etoposilone B alone (S_0), i.e. , D^* , used to calculate the curves relative to overlapping additivity for A549 cells, were 2.3 and 1.6 Gy for photons and protons respectively. The correspondent values for U251MG cells were 2.4 and 1.5 Gy.

For both the cell lines and both the radiation types, the experimental curves relative to the combined treatment fall below the additivity region bounded by the two calculated curves, thus indicating a synergism between the two agents, but the effect is more marked for photons than protons.

In order to quantify the effectiveness of the combined treatment compared with irradiation alone, the Dose Enhancement Factor (DEF) was evaluated. The DEF is defined as the ratio between the radiation doses used alone and in conjunction with the drug to obtain the same biological end point (*i.e.* the same survival level). For A549 cells, at 10% of survival, DEF resulted 1.5 ± 0.1 and 1.2 ± 0.1 for photons and protons respectively; for U251MG cells it resulted 1.6 ± 0.2 and 1.4 ± 0.1 for photons and protons, respectively.

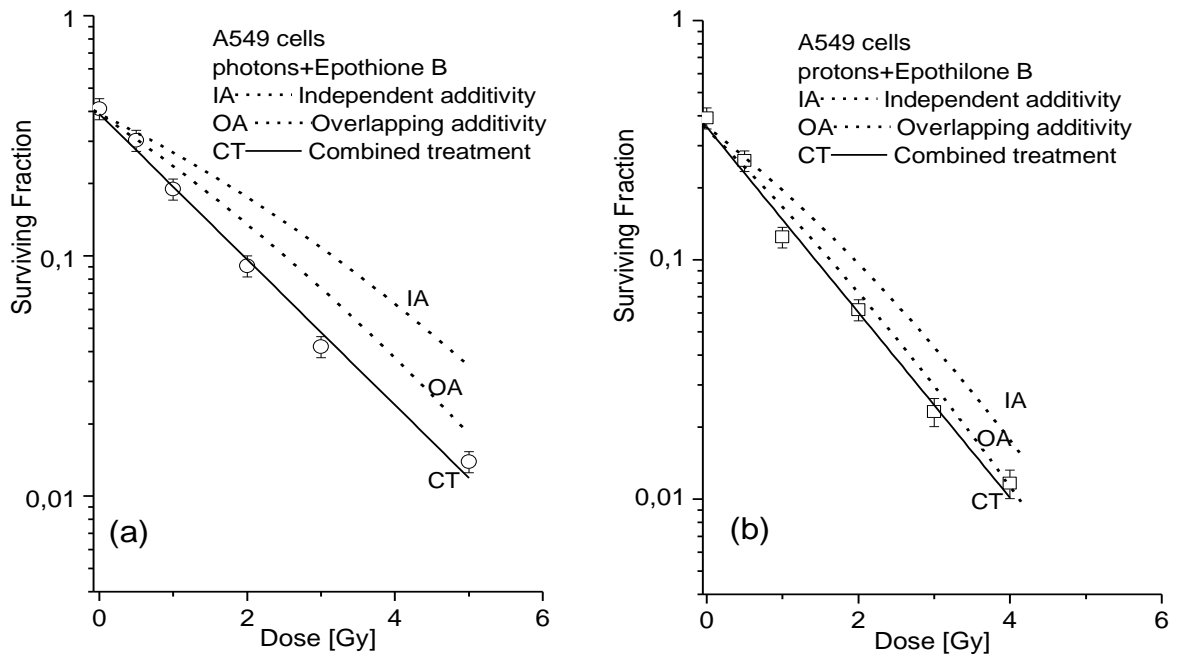


Fig. 4 Analysis of radiation-drug interaction in A549 cells. Experimental data relative to photon (a) and proton (b) irradiation in conjunction with 0.075 nM Epothilone B are reported and fitted with the solid line (CT). The dashed line (IA) represents drug and radiation acting independently on cell survival. The dashed line (OA) is relative to the overlapping additivity.

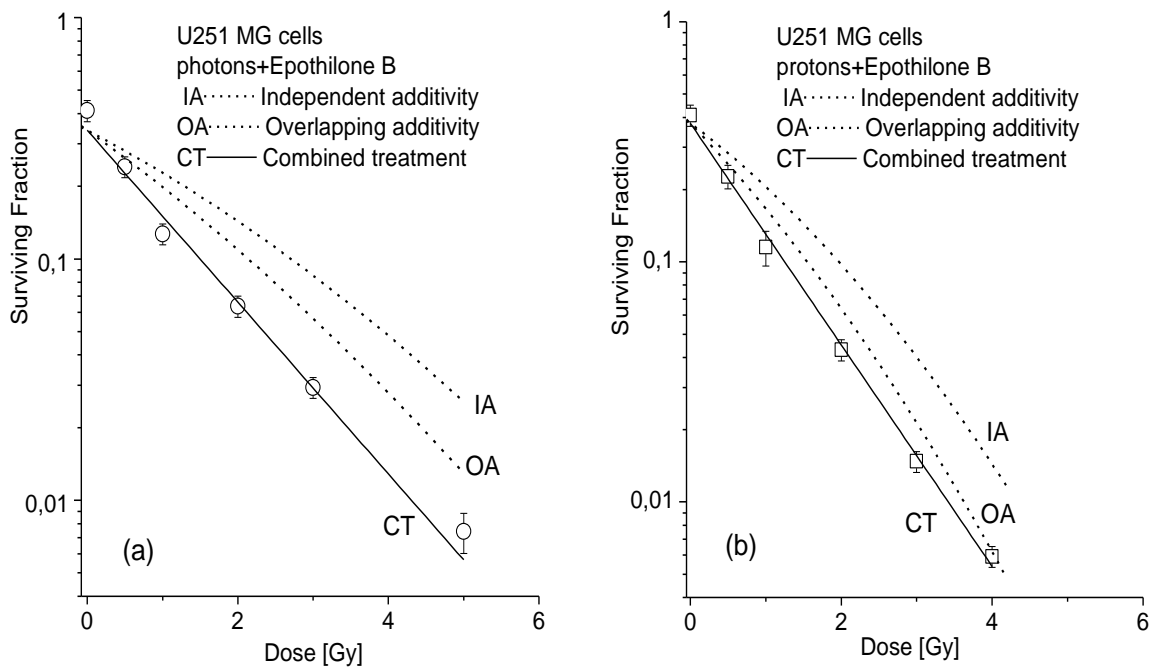


Fig. 5 Analysis of radiation-drug interaction in U251 MG cells. Experimental data relative to photon (a) and proton (b) irradiation in conjunction with 0.125 nM Epothilone B are reported and fitted with the solid line (CT). The dashed line (IA) represents drug and radiation acting independently on cell survival. The dashed line (OA) is relative to the overlapping additivity.

4. Discussion

We investigated the response of lung adenocarcinoma cells A549 and glioblastoma U251MG to irradiation with a SOBP proton beam alone and in combination with Etoposide. The same study was performed on a 6 MV photon beam. The results show that Etoposide has an anticlonogenic effect at subnanomolar concentrations in both A549 and U251MG cells and that its use modifies the response of these cells to photon and proton irradiation, removing the typical shoulder of the dose-survival curves found after treatment with radiation alone. The interaction modality between photons or protons and Etoposide in A549 and U251MG cells is slightly synergistic. Our results with photon beam on A549 cells are in agreement with those of Baumgart (Baumgart *et al.* 2012), who found for the same cell line a synergistic effect of Etoposide used in combination with photon beams irradiation. Furthermore Roher-Bley (Roher-Bley *et al.* 2009) and Roher-Bley (Roher-Bley *et al.* 2013) found an at least additive effect of Etoposide and X-rays on A549 cell line. Hofstetter (Hofstetter *et al.* 2005) observed that Etoposide has a radiosensitizing effect in the human colon adenocarcinoma cell line SW480 and in p53-null MEF cells when combined with X-rays. Oehler (Oehler *et al.* 2011) demonstrated that Etoposide is a very effective cytotoxic agent against several medulloblastoma cell lines, that it strongly reduces clonogenic survival alone and in conjunction with ionizing radiation at picomolar concentrations. It also resulted in an at least additive anticlonogenic effect in combination with clinically relevant dose of X rays.

As far as we know there are no published studies on the use of Etoposide in combination with proton beam irradiation on A549 or U251MG cells. The results of the present study show that Etoposide increases protons cytotoxicity with a synergism which is slightly weaker than that of photons, as shown by the DEF values, greater for photons than protons. This weaker synergism may be due to different amounts of reparable damages induced by the two radiation types (greater for photons than protons). In fact, we found that proton RBE values at 10% of survival are 1.5 ± 0.2 for both A549 and U251MG cells. These RBE values are higher than 1.1, value which is conventionally used for therapeutic proton beams (ICRU report 2007). *In vitro* values of proton RBE from literature show significant variations as reported in some reviews (Paganetti 2014, Jones 2016). These RBE variations may be due to different experimental conditions (beam energy, active scanning techniques or passive beam modulation, methods

for RBE calculation, cell lines, reference radiation). Furthermore data emerging from recent studies suggest that for several end points the biological response is differentially modulated by protons compared to photons (Tommasino and Durante 2015, Girdhani *et al* 2013). As shown by Girdhani (Girdhani *et al* 2013), several reports have suggested that at clinically relevant energies, protons can be more effective than photons to produce DSBs (DNA double-strand-breaks). Calugaru (Calugaru *et al* 2011) showed that the incidence of DSBs and clustered lesions was higher for protons than for ¹³⁷CS gamma-rays. Britten (Britten *et al* 2013) measured RBE values for cell killing of Hep2 human laryngeal cancer cells at various positions along the depth dose profile of modulated proton therapy beams. The authors concluded that the RBE for human cancer cells in the mid-distal part of the SOBP is greater (1.5-2.1) than the widely used 1.1 value. Recently, Mitteer (Mitteer *et al* 2015) using glioma stem cells found that, compared to photons, proton beams induces greater DNA damage, cell cycle alteration and cytotoxicity through reactive oxygen species (ROS). The difference in the amount of correctly-reparable damage induced by photon or proton irradiation may explain not only the high proton RBE values found in the present study, but also the weaker synergism found for the interaction of protons, compared to photons, with Etoposide B. Indeed it was demonstrated (Baumgart *et al* 2012), (Baumgart *et al* 2015), that Etoposide B reduces DNA repair capability of tumour cells, a property which may be less relevant in the case of interaction of the drug with a radiation inducing less reparable lesions. Experiments on the interaction of Etoposide B with Carbon-ion beams which, compared with protons, induce even less reparable damage due to their higher ionization density, are in progress in our Laboratory to investigate this aspect. Despite the weak synergism, proton beams are very promising to be used in combination with a non tumour-specific drug (such as Etoposide B), due to their good conformability to the tumour volume.

5. Conclusion

In conclusion, our *in vitro* study shows that Etoposide B has a radiosensitizing effect in A549 and U251MG cells when combined with proton or photon beams, with a synergistic type of interaction with radiation. The results of the present study provide a radiobiological basis for further experiments and

suggest that the combination of Etoposide B with protontherapy could be tested in controlled phase I clinical trials in advanced NSCLC and GM patients as a new and promising tool for treatment.

References

- Agrawal N R, Ganapathi R, Mekhail T 2003 Tubulin interacting agents: novel taxanes and etoposides *Curr Oncol Rep* **5** 89-98.
- Albain K S *et al* 2009 Radiotherapy plus chemotherapy with or without surgical resection for stage III non-small-cell lung cancer: a phase II randomised controlled trial *The Lancet* **374** 379-86.
- Altmann K H and Gertsch J 2007 Anticancer drugs from nature-natural products as unique source of new microtubule-stabilizing agents *Nat Prod Rep* **24** 327-57.
- Altmann K H, Wartmann M, O'Reilly T 2000 Etoposides and related structures-a new class of microtubule inhibitors with potent in vivo antitumour activity *Biochim Biophys Acta* **1470** M79-M91.
- Bao Y *et al* 2015 Phase II trial of recombinant human endostatin in combination with concurrent chemoradiotherapy in patients with stage III non-small-cell lung cancer *Radiother Oncol* **114** 161-66.
- Barazzuol L, Jena R, Burnet N G, Jeynes J C G, Merchant M J, Kirkby K J, Kirkby N F 2012 In vitro evaluation of combined Temozolomide and Radiotherapy using X rays and High-Linear Energy Transfer Radiation for Glioblastoma. *Rad Res* **177** 651-62.
- Baumgart T, Klautke G, Kriesen S, Kuznetsov S A, Weiss D G, Fietkau R, Hildebrandt G, Manda K 2012 Radiosensitizing effect of Etoposide B on human epithelial cancer cells *Strahlenther Onkol* **188** 177-84.
- Baumgart T, Kriesen S, Neels O, Hildebrandt G., Manda K 2015 Investigation of Etoposide B-Induced Cell Death Mechanisms in Human Epithelial Cancer Cells –in Consideration of Combined Treatment With Ionizing Radiation *Cancer Investig* **33** 213-24.
- Bocci G, Nicolaou K C, Kerbel R S 2002 Protracted low-dose effects on human endothelial cell proliferation and survival in vitro reveal a selective antiangiogenic window for various chemotherapeutic drugs *Cancer Res* **62** 6938-43.
- Bollag D M, McQueney P A, Zhu J, Hensens O, Koupal L, Liesch J, Goetz M, Lazarides E, Woods C M 1995 Etoposides a new class of microtubule-stabilizing agents with a taxol-like mechanism of action *Cancer Res* **55** 2325-33.
- Britten R A, Nazaryan V, Davies L K, Klein S B, Nichiporov D, Mendonca M S, Wolanski M, Nie X, George J, Keppel C 2013 Variations in the RBE for cell killing along the depth-dose profile of modulated proton therapy beam *Radiat Res* 2013 **179** 21-28.
- Calugaru V, Nauraye C, Noel G, Giocanti N, Favaudon V, Megnin-Chanet F 2011 Radiobiological characterization of two therapeutic proton beams with different initial Energy spectra used at the institut Curie Proton Therapy Center in Orsay *Int J Radiat Oncol Biol Phys* **81** 1136-43
- Chalmers A J, Ruff E M, Martindale C, Lovegrove N, Short S C 2009 Cytotoxic effects of Temozolomide and radiation are additive and schedule-dependent *Int J Radiat Oncol Biol Phys* **75** 1511-19

- Chang J Y, Komaki R, Lu C, Wen H Y, Allen P K, Tsao A, Gillin M, Mohan R, Cox J D 2011 Phase 2 study of high-dose proton therapy with concurrent chemotherapy for unresectable stage III non small cell lung cancer *Cancer* **117** 4707-13.
- Combs S E, Zipp L, Rieken S, Habermehl D, Brons S, Winter M, Haberer T, Debus J and Weber K J 2012 *In vitro* evaluation of photon and carbon ion radiotherapy in combination with chemotherapy in glioblastoma cells *Radiat Oncol* **7** 9.
- Combs S E *et al* 2010 Randomized phase II study evaluating a carbon ion boost applied after combined radiotherapy with Temozolomide versus a proton boost after radiochemotherapy with Temozolomide in patients with primary glioblastoma: the CLEOPATRA trial *BML Cancer* **10** 478-86.
- Durante M. 2014 New challenges in high-energy particle radiobiology *Br J Radiol* **87** 20130626.
- Eberhardt W E E 2015 Concurrent chemoradiotherapy in stage III non-small-cell lung cancer: what is the best regimen? *J Clin Oncol* **33** 532-33.
- El Shafie R A, Habermehl D, Rieken S, Mairani A, Orschiedt L, Brons S, Haberer T, Weber KJ, Debus J, Combs S E 2013 *In vitro* evaluation of photon and raster-scanned carbon ion radiotherapy in combination with gemcitabine in pancreatic cell lines *J Radiat Res* **54** 113-19.
- Ferlay J, Parkin D M, Steliarova-Foucher E 2010 Estimates of cancer incidence and mortality in Europe in *Eur J Cancer* **46** 765-81.
- Ferretti S, Allegrini P R, O'Reilly T, Schnell C, Stumm M, Wartmann M, Wood J, McSheehy P M 2005 Patupilone induced vascular disruption in orthotopic rodent tumor models detected by magnetic resonance imaging and interstitial fluid pressure *Clin Cancer Res* **11** 7773-84.
- Fitzek M M *et al* 1999 Accelerated fractionated proton/photon irradiation to 90 cobalt gray equivalent for Glioblastoma multiforme: results of a phase II prospective trial *J Neurosurg* **91** 251-60
- Fogh S, Machtay M, Werner-Wasik M, Curran W J Jr, Bonanni R, Axelrod R, Andrews D, Dicker A P 2010 Phase I trial using Patupilone (Epothilone B) and concurrent radiotherapy for central nervous system malignancies *Int J Radiat Oncol Biol Phys* **77** 1009-16.
- Furmanova-Hollenstein P, Broggin-Tenzer A, Eggel M, Millard A L, Pruschy M 2013 The microtubule stabilizer Patupilone counteracts ionizing radiation-induced matrix metalloproteinase activity and tumor cell invasion. *Radiat Oncol* **8** 105.
- Girdhani S, Sachs R, Hlatky L 2013 Biological effects of proton radiation: what we know and don't know *Radiat Res* **179** 257-72.
- Grutters JP, Kessels AG, Pijls-Johannesma M, De Ruyscher D, Joore MA, Lambin P 2010 Comparison of the effectiveness of radiotherapy with photons, protons and carbon-ions for non-small cell lung cancer: A meta-analysis *Radiother Oncology* **95** 32-40.
- Hall EJ and Giaccia AJ 2006 *Radiobiology for the Radiologist* 6th Edition Lippincott Williams and Wilkins Philadelphia 30-32.
- Hofstetter B, Vuong V, Broggin-Tenzer A, Bodis S, Ciernik IF, Fabbro D, Wartmann M, Folkers G, Pruschy M 2005 Patupilone acts as radiosensitizing agent in multidrug-resistant cancer cells in vitro and in vivo *Clin Cancer* **11** 1588-96.
- International Atomic Energy Agency 2010 *Radiation Biology: A Handbook for Teachers and Students* Vienna (Training Course Series 42) 28.

- International Commission on Radiation Units and Measurements 2007 Prescribing, recording and reporting proton-beam therapy (ICRU Report 78) *J ICRU* **7** 1-210.
- Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun M J 2009 Cancer statistic *C A Cancer J Clin* **59** 225-49.
- Jones B 2016 Why RBE should be a variable and not a constant in proton therapy *Br J Radiol* **89** 20160116.
- Kim J C, Kim J S, Saha D, Cao Q, Shyr Y, Choy H 2005 Potential radiation-sensitizing effect of semisynthetic Etoposide B in human lung cancer cells *Radiother Oncol* **68** 305-13.
- Kitabayashi H, Shimada H, Yamada S, Yasuda S, Kamata T, Ando K, Tsujii H, Ochiai T 2006 Synergistic growth suppression induced in esophageal squamous cell carcinoma cells by combined treatment with docetaxel and heavy carbon-ion beam irradiation *Oncol Rep* **15** 913-18.
- Liao Z, Lin S H, Cox J D 2011 Status of particle therapy for lung cancer *Acta Oncol* **50** 745-56.
- Loeffler J S and Durante M 2013 Charged particle therapy – optimization, challenges and future directions *Nat Rev Clin Oncol* **10** 411-24.
- Luttjeboer M, Lafleur M V, Kwidama Z J, Van Rijn J, Van Den Berg J, Slotman B J, Kaspers G J, Cloos J 2010 Strategies for the analysis of in vitro radiation sensitivity and prediction of interaction with potential radiation modifying agents *Int J Radiat Biol* **86** 458-66.
- Mirandola A, Molinelli S, Vilches Freixas G, Mairani A, Gallio E, Panizza D, Russo S and Ciocca M 2015 Dosimetric commissioning and quality assurance of scanned ion beams at the Italian National Center for Oncological Hadrontherapy. *Med Phys* **42** 5287.
- Mirmanoff R O *et al* 2006 Radiotherapy and Temozolomide for newly diagnosed glioblastoma: recursive partitioning analysis of the EORTC 26981/22981 – NCIC CE3 phase III randomized trial *J Clin Oncol* **24** 2563-69.
- Mitteer RAJ, Wang Y, Shah J, Gordon S, Fager M, Butter PP, Kim HJ, Guardiola-Salmeron C, Carabe-Fernandez A and Fan Y. Proton beam radiation induces DNA damage and cell apoptosis in glioma stem cells through reactive oxygen species. *Sci Rep* 2015; 5:13961.
- O'Rourke N, Roqué i Figuls M, Farré Bernadó N, Macbeth F 2010 Concurrent chemoradiotherapy in non-small cell lung cancer (Review) *The Cochrane Library* Issue **6** John Wiley & Sons.
- Oehler C *et al* 2012 Patupilone for recurrent glioblastoma: clinical outcome and translational analysis of a single-institution phase I/II trial *Oncol* **83** 1-9.
- Oehler C, von Bueren A O, Furmanova P, Brogini-Tenzer A, Orłowski K, Rutkowski S, Frei K, Grotzer M A, Pruschy M 2011 The microtubule stabilizer patupilone (Etoposide B) is a potent radiosensitizer in medulloblastoma cells *Neuro Oncol* **13** 1000-10.
- O'Reilly T, Wartmann M, Brueggen J, Allegrini P R, Floersheimer A, Maira M, McSheehy P M 2008 Pharmacokinetic profile of the microtubule stabilizer patupilone in tumor-bearing rodents and comparison of anti-cancer activity with other MTS in vitro and in vivo *Cancer Chemother Pharmacol* **62** 1045-54.
- Paganetti H and Van Luijk P 2013 Biological considerations when comparing proton therapy with photon therapy *Semin Radiat Oncol* **23** 77-87.

- Paganetti H 2014 Relative biological effectiveness (RBE) values for proton beam therapy. Variations as a function of biological endpoint, dose, and linear energy transfer. *Phys Med Biol* **59** R419-R472.
- Pagano A, Honorè S, Mohan R, Berges R, Akhmanova A, Braguer D 2012 Epothilone B inhibits migration of glioblastoma cells by inducing microtubule catastrophes and affecting EB1 accumulation at microtubule plus ends *Biochemical Pharmacology* **84** 432-43.
- Pawlik TM and Keyomarsi K 2004 Role of cell cycle in mediating sensitivity to radiotherapy *Int J Radiat Oncol Biol Phys* **59** 928-42.
- Rieken S, Habermehl D, Haberer T, Jaekel O, Debus J, Combs S E 2012 Proton and Carbon ion radiotherapy for primary brain tumors delivered with active raster scanning at the Heidelberg Ion Therapy Center (HIT): early treatment results and study concepts *Radiat Oncol* **7** 41-7.
- Rohrer Bley C, Furmanova P, Orłowski K, Grosse N, Broggin-Tenzer A, McSheehy P M, Pruschy M. 2013 Microtubule stabilising agents and ionising radiation: multiple exploitable mechanisms for combined treatment *Eur J Cancer* **49** 245-53.
- Rohrer Bley C, Jochum W, Orłowski K, Furmanova P, Vuong V, McSheehy P M, Pruschy M 2009 Role of the microenvironment for radiosensitization by Patupilone *Clin Cancer Res* **15** 1335-42.
- Rossi S 2015 The National Centre for Oncological Hadrontherapy (CNAO): Status and perspectives *Phys Med* **31** 333-51.
- Schlaich F, Brons S, Haberer T, Debus J, Combs S E and Weber K J 2013 Comparison of the effects of photon versus carbon ion irradiation when combined with chemotherapy in vitro. *Radiat Oncol*; **8** 260.
- Stupp R *et al* 2009 Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomized phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol* **10** 459-66.
- Stupp R *et al* 2005 Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma *N Engl J Med* **352** 987-96.
- Suit H, Kooy H, Trofimov A, Farr J, Munzenrider J, DeLaney T, Loeffler J, Clasie B, Safai S, Paganetti H 2008 Should positive phase III clinical trial data be required before proton beam therapy is more widely adopted? No *Radiother Oncol* **82** 148-53.
- Tommasino F and Durante M 2015 Proton Radiobiology *Cancers* **7** 353-81.
- Vansteenkiste J, *et al* 2007 Phase II clinical trial of the Epothilone B analog, ixabepilone, in patients with non-small-cell lung cancer whose tumors have failed first-line platinum-based chemotherapy *J Clin Oncol* **25** 3448-55.
- Woltering E A, Lewis J M, Maxwell P J, Frey D J, Wang Y, Rothermel J, Anthony C T, Balster D A, O'Leary J P and Harrison L H. 2003 Development of a novel in vitro human tissue-based angiogenesis assay to evaluate the effect of antiangiogenic drugs *Annals of Surgery* **237** 790-800.