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

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**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 Epothilone B 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 Epothilone B 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 Epothilone B at subnanomolar concentration has an anticlonogenic effect. Furthermore, differently from the results found with radiation alone, the survival curves for the combined treatment Epothilone B - 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 chemotherapeutical agent for brain malignancies. Epothilone B has shown antivascular 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 consolidate 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% $CO_2$ 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 then 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 $cm^2$ 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 $cm^2$ 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 Epothilone 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 Epothilone 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 clogenic 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 ).

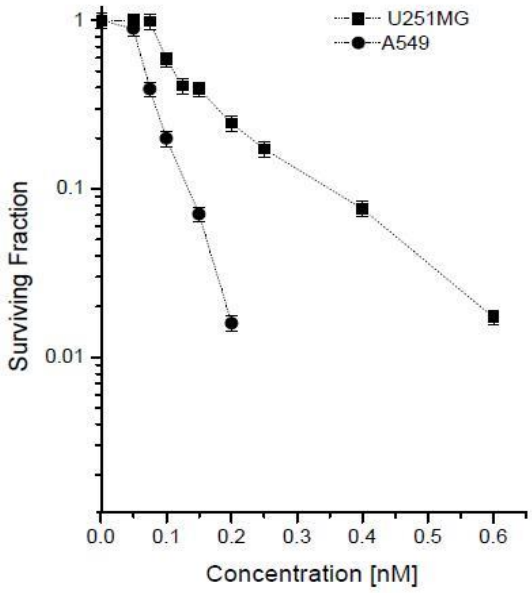


**Fig. 1** Surviving fraction of A549 (•) and U251MG (■) cells as a function of Epothilone B concentration. Error bars represent SE (SE = Standard Error**).**

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 Epothilone B, protons effectiveness relative to combined treatments with photons can be evaluated as the ratio of the slopes of the two exponential survival curves ($\alpha_p/\alpha_X$). 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.

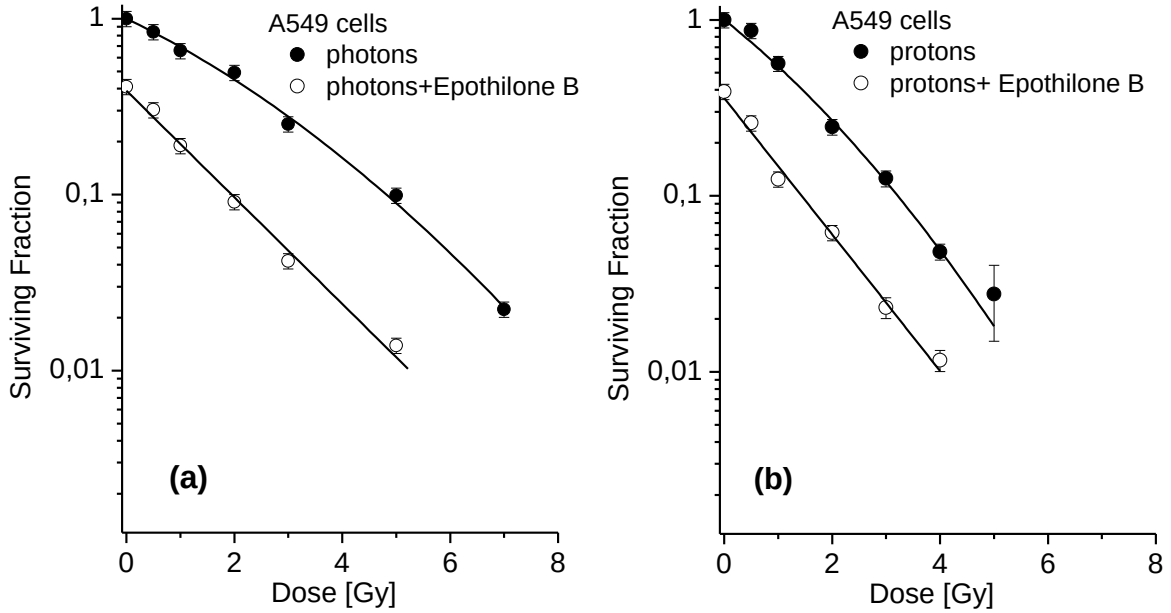


Fig. 2 Surviving fraction (mean of 4 independent experiments) of A549 cells exposed to (a) photon and (b) proton beams alone (●) and in combination with 0.075 nM Epothilone B (○). Solid lines are the fit of the experimental data according to eqs (1) or (2) for radiation alone or combined with Epothilone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

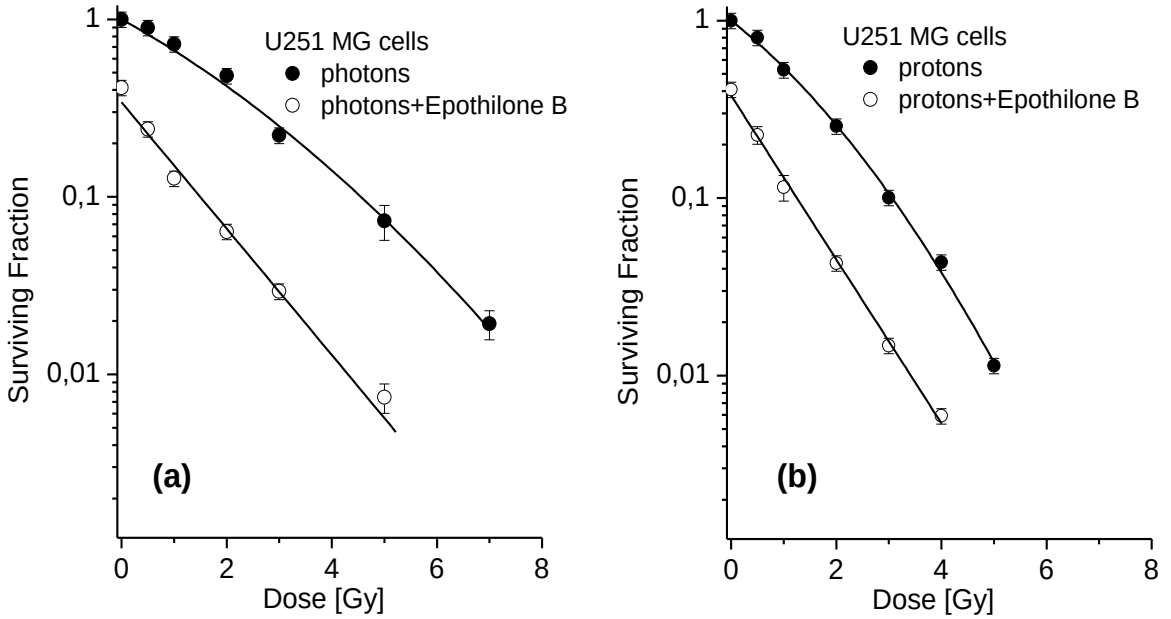


Fig.3 Surviving fraction (mean of 4 independent experiments) of U251MG cells exposed to (a) photon and (b) proton beams alone (●) and in combination with 0.125 nM Epothilone B (○). Solid lines are the fit of the experimental data according to eqs (1) or (2) for radiation alone or combined with Epothilone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

**Table 1: Parameters of Survival Curves**

| | | Radiation alone | | Radiation + Epothilone B | |
|---|---|---|---|---|---|
| Radiation | Cell line | $\alpha \pm SE$ [$Gy^{-1}$] | $\beta \pm SE$ [$Gy^{-2}$] | $S_0 \pm SE$ | $\alpha \pm SE$ [$Gy^{-1}$] |
| 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 Epothilone B according to eqs (1) or (2) respectively. $S_0$ is the clonogenic survival of cells treated with Epothilone B alone. SE= Standard Error.

3.3 *Interaction between radiation and Epothilone 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 Epothilone 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.

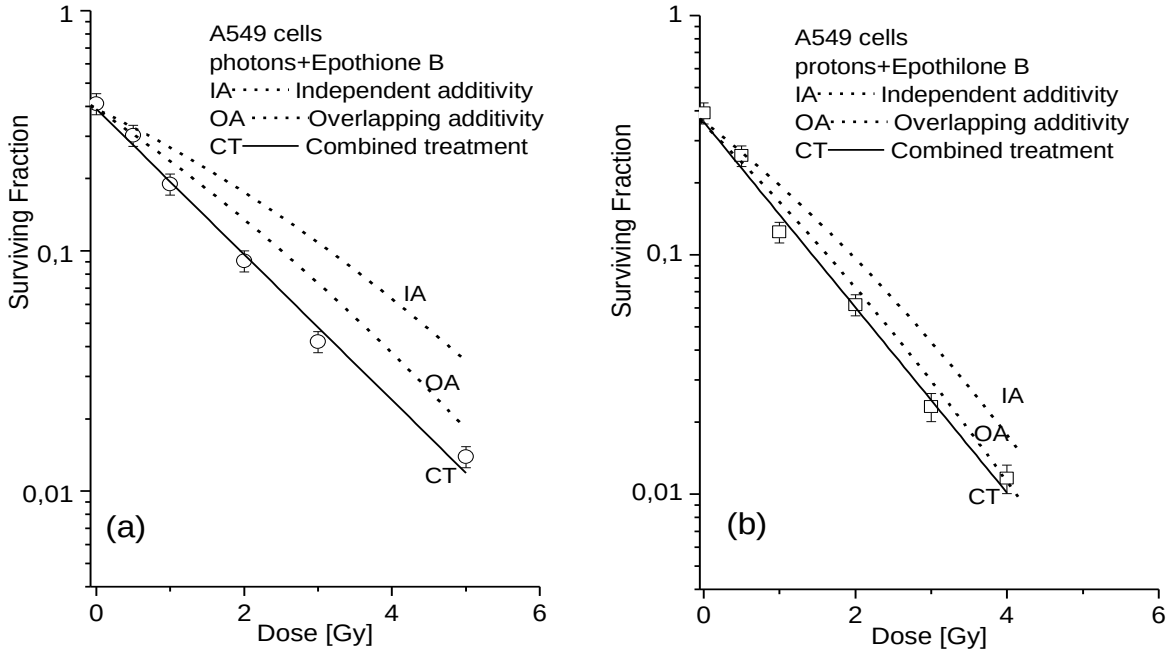


**Fig. 4** Analysis of radiation-drug interaction in A549 cells. Experimental data relative to photon (a) and proton (b) irradiation in conjunction with 0.075 nM Epothilone B are reported and fitted with the solid line (CT). The dashed line (IA) represents drug and radiation acting independently on cell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

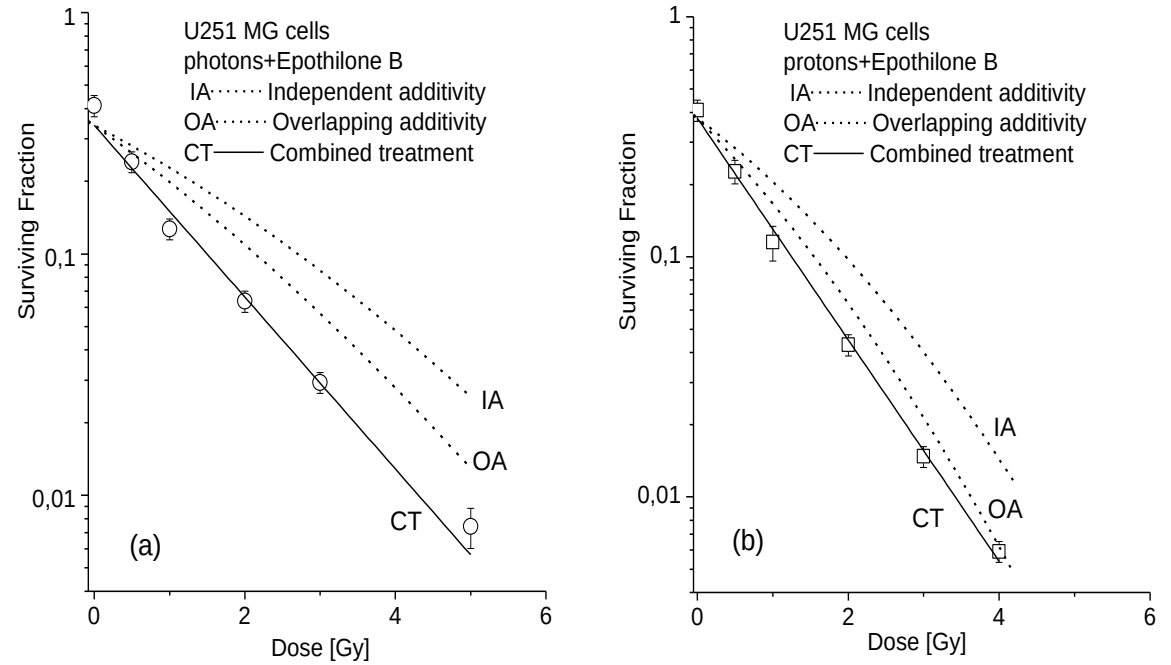


**Fig. 5** Analysis of radiation-drug interaction in U251 MG cells. Experimental data relative to photon (a) and proton (b) irradiation in conjunction with 0.125 nM Epothilone B are reported and fitted with the solid line (CT). The dashed line (IA) represents drug and radiation acting independently on 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 Epothilone B. The same study was performed on a 6 MV photon beam. The results show that Epothilone B 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 Epothilone B 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 Epothilone B 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 Epothilone B and X-rays on A549 cell line. Hofstetter (Hofstetter *et al* 2005) observed that Epothilone B has a radiosenziting 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 Epothilone B 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 Epothilone B in combination with proton beam irradiation on A549 or U251MG cells. The results of the present study show that Epothilone B 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 $^{137}$CS 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 Epothilone B. Indeed it was demonstrated (Baumgart *et al* 2012), (Baumgart *et al* 2015), that Epothilone 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 Epothilone 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 Epothilone B), due to their good conformability to the tumour volume.

## 5. Conclusion

In conclusion, our *in vitro* study shows that Epothilone 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 Epothilone 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.

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