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## Indirect radio-chemo-beta therapy: a targeted approach to increase biological efficiency of x-rays based on energy

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### Abstract

Despite the use of multimodal treatments incorporating surgery, chemotherapy and radiotherapy, local control of gliomas remains a major challenge. The potential of a new treatment approach called indirect radio-chemo-beta therapy using the synergy created by combining methotrexate (MTX) with bromodeoxyuridine (BrUdR) under optimum energy x-ray irradiation is assessed. 9L rat gliosarcoma cells pre-treated with 0.01  $\mu\text{M}$  MTX and/or 10  $\mu\text{M}$  BrUdR were irradiated in vitro with 50 kVp, 125 kVp, 250 kVp, 6 MV and 10 MV x-rays. The cytotoxicity was assessed using clonogenic survival as the radiobiological endpoint. The photon energy with maximum effect was determined using radiation sensitization enhancement factors at 10% clonogenic survival (SER10%). The cell cycle distribution was investigated using flow cytometric analysis with propidium iodide staining. Incorporation of BrUdR in the DNA was detected by the fluorescence of labelled anti-BrUdR antibodies. The radiation sensitization enhancement exhibits energy dependence with a maximum of 2.3 at 125 kVp for the combined drug treated cells. At this energy, the shape of the clonogenic survival curve of the pharmacological agents treated cells changes substantially. This change is interpreted as an increased lethality of the local radiation environment and is attributed to supplemented inhibition of DNA repair. Radiation induced chemo-beta therapy was demonstrated in vitro by the targeted activation of combined pharmacological agents with optimized energy tuning of x-ray beams on 9 L cells. Our results show that this is a highly effective form of chemo-radiation therapy.

### Keywords

rays, x, efficiency, biological, increase, approach, energy, targeted, indirect, therapy, beta, chemo, radio

### Disciplines

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# Indirect radio-chemo-beta therapy: a targeted approach to increase biological efficiency of X-rays based on energy

**Running heads:** Indirect Radio-Chemo-Beta therapy

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## Abstract

Despite the use of multimodal treatments incorporating surgery, chemotherapy and radiotherapy, local control of gliomas remains a major challenge. The potential of a new treatment approach called indirect radio-chemo-beta therapy using the synergy created by combining methotrexate (MTX) with bromodeoxyuridine (BrUdR) under optimum energy X-ray irradiation is assessed. 9L rat gliosarcoma cells pre-treated with 0.01  $\mu\text{M}$  MTX and/or 10  $\mu\text{M}$  BrUdR were irradiated *in vitro* with 50 kVp, 125 kVp, 250 kVp, 6 MV and 10 MV X-rays. The cytotoxicity was assessed using clonogenic survival as the radiobiological endpoint. The photon energy with maximum effect was determined using radiation sensitization enhancement factors at 10% clonogenic survival ( $\text{SER}_{10\%}$ ). The cell cycle distribution was investigated using flow cytometric analysis with propidium iodide staining. Incorporation of BrUdR in the DNA was detected by the fluorescence of labeled anti-BrUdR antibodies. The radiation sensitization enhancement exhibits energy dependence with a maximum of 2.3 at 125 kVp for the combined drug treated cells. At this energy, the shape of the clonogenic survival curve of the pharmacological agents treated cells changes substantially. This change is interpreted as an increased lethality of the local radiation environment and is attributed to supplemented inhibition of DNA repair. Radiation induced chemo-beta therapy was demonstrated *in vitro* by the targeted activation of combined pharmacological agents with optimized energy tuning of X-ray beams on 9L cells. Our results show that this is a highly effective form of chemo-radiation therapy.

**Keywords:** Multimodal therapy, high-Z, radioresistant, brain cancer, methotrexate, bromodeoxyuridine

## 1. Introduction

Gliomas are among the most frequent primary brain tumours in adults, with an incidence of approximately 5–11/100,000 among the population per year in industrial countries. The advent of

imaging techniques has made early diagnosis possible and recently there has been some improvement in treatment techniques leading to increases in the median 5-year survival of patients (Malmstrom *et al.* 2012; Scott *et al.* 2011; Stupp *et al.* 2009). However, the treatment of high-grade gliomas remains a major challenge and is currently palliative rather than curative. Despite different multimodal treatments, including surgery, chemotherapy and radiotherapy, these gliomas remain extremely radioresistant while the surrounding normal tissues are comparably radiosensitive, especially in children.

Although dose enhancement improves local control of tumours, it is limited by the tolerance of healthy tissue. One of the main reasons for radiotherapy failure is post-treatment tumour regrowth. Consequently improved therapeutic ratios are needed. These rely on the development of new mechanisms to increase the X-ray dosage in tumours while sparing the surrounding tissues. Similarly, adjuvant chemotherapy generally fails to improve patient outcomes because of inadequate drug delivery inside the tumour (Behin *et al.* 2003; Bredel 2001). Considerable efforts have been made to optimize chemo-radiotherapy treatments of tumours by increasing both the chemotherapeutic drug concentration and the radiation dose while minimising the effect on healthy tissues (Behin *et al.* 2003). Linking drug activation to an optimized energy tuning of X-ray beams could permit a highly effective chemo-radiation therapy that significantly limits drug and radiation toxicity for healthy tissues.

Many anticancer drugs act on nucleotide biosynthesis to destroy rapidly cycling tumour cells. For example, they may inhibit enzymes involved in tetrahydrofolate synthesis such as dihydrofolate reductase (DHFR), or in thymidine synthesis such as thymidylate synthetase (TS). The well-known antimetabolite chemotherapeutic agents methotrexate (MTX), 5-Fluorouracil (5FU) and raltitrexed break the thymidylate synthesis cycle by respectively blocking DHFR (Huennekens 1994; Tehei *et al.* 2006) or TS enzymes (Pinedo *et al.* 1988). The folate analogue MTX is taken up in the cell by folate receptor-mediated endocytosis and it has a high affinity for those receptors. The cell surface receptor for folate is overexpressed in a large number of tumours including brain cancer. As the expression of folate receptors is low in normal cells, MTX has a high selectivity for brain tumours and does not normally accumulate in healthy tissue (Lu *et al.* 2003; Wang *et al.* 1998; Weitman *et al.* 1992).

Halogenated pyrimidine analogues, such as bromodeoxyuridine (BrUdR) or iododeoxyuridine (IUdR), are radiosensitising drugs which compete with thymidine for incorporation into the structure of DNA. Consequently, a halogenated compound (containing bromine  $Z=35$  or iodine  $Z=53$ ) can be introduced directly into DNA - this is the reason why such compounds are strong candidates for both radioactive Auger emitter (Kassis *et al.* 1982; Kassis *et al.* 1998) and synchrotron radiation-induced Auger cascade studies (Corde *et al.* 2004; Laster *et al.* 1993; Usami *et al.* 1991). These latter *in vitro* studies have demonstrated a significant increase in the Auger enhancement ratio, up to a factor of three, for synchrotron X-ray irradiation above the iodine K-absorption edge (Laster *et al.* 1993) and for a 100kVp x-ray beam (Karnas *et al.* 1999). Useful radiosensitization with thymidine analogues depends on achieving significant uptake into the cancer cell DNA and minimising the number of cells that have no thymidine replacement. MTX and 5FU inhibition of the TS cycle has been shown to increase the incorporation of bromine or iodine thymidine analogues into cells (Benson *et al.* 1985; Bruce-Gregorios *et al.* 1991; Kassis *et al.* 2000).

Earlier synchrotron radiation study in the keV energy range using halogenated component revealed that the dose enhancement is dependent on the beam energy, with an optimum therapeutic irradiation based on the mass energy-absorption coefficient of the target due to the presence of halogenated component (Corde *et al.* 2004). Conventional kilovoltage and megavoltage X-ray beams are widely used for cancer radiotherapy treatment and provide a broader irradiation energy range. In the megavoltage range of energies (< 20 MeV), photon beams mainly interact with matter by Compton scattering and pair production processes. These processes can produce high-energy electrons with a long-range energy deposition. In the kilovoltage range, the demonstrated dose enhancement is mainly due to the photoelectric effect. The resulting Auger electrons have a shorter range and can deposit energy with greater radiobiological effectiveness due to a high linear energy transfer in aqueous solution (Corde *et al.* 2004).

In this work, we investigate a new modality for radioresistant brain tumour treatment called indirect radio-chemo-beta therapy using the synergy of the chemotherapy drug methotrexate, the halogenated pyrimidine bromodeoxyuridine containing a high Z (atomic number) atom and X-ray radiation beams. We study the optimum energy of our treatment by probing the radiation sensitization enhancement factor along the energy spectrum from 50 kVp to 10 MV. Using clonogenic survival as our radiobiological endpoint, the radiation sensitization enhancement is shown to be energy dependent, with a maximum at 125 kVp for cells pre-treated with the combined drugs. The experimental optimal energy value of the enhancement ratio produced in the presence of the BrUdR agrees with theoretical predictions (Corde *et al.* 2004). The methotrexate enhances radiation sensitization by inhibiting production of the endogenous substrate dTMP and therefore decreasing DNA repair. The associated cell survival curves demonstrate a significant change in shape, indicative of increased lethality of the local radiation environment possibly instigated by the inhibition of the DNA repair. Contributing mechanisms for the observed increased lethality have been investigated using flow cytometry and are discussed. Bromide incorporation was quantified using immunocytochemical staining of BrUdR. Our data obtained in this study emphasizes the importance of linking drug activation to a precise targeting of optimal energy X-ray beams. This permits a highly effective chemo-radiation therapy that significantly limits drug and radiation toxicity for healthy tissues.

## **2. Materials and Methods**

### *2.1. Cell culture*

9L rat gliosarcoma cell line derived from N-nitrosomethylurea-induced tumor was obtained from the European Collection of Cell Cultures (ECACC). Cells were maintained in exponential growth in DMEM (Invitrogen, AU) with L-Glutamine and supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Pen Strep) at 37°C in a humidified incubator with 5% CO<sub>2</sub> in air.

### *2.2. Drugs treatment and cytotoxicity*

Methotrexate (MTX) and 5-bromo-2'-deoxyuridine (BrUdR) (Sigma-Aldrich, MO, USA) were prepared in Hanks' balanced salt solution and stored at -20°C. 9L cells seeded in T12.5 cm<sup>2</sup> flasks in complete DMEM were allowed to attach and divide for 48 hours. Then, medium was replaced

and cells were incubated with 0.01  $\mu\text{M}$  MTX and/or 10  $\mu\text{M}$  BrUdR. After a two doubling times incubation with the drugs, the cytotoxic effect of drugs was determined by clonogenic assay.

### 2.3. Irradiation procedures

Confluent cell cultures were irradiated in T12.5  $\text{cm}^2$  flasks following a two doubling times incubation period with drugs. Prior to irradiation, the flasks were completely filled with drugs-containing medium (drugs concentration maintained). The Nucletron Oldelft Therapax DXT 300 Series 3 Orthovoltage unit (Nucletron B.V., Veenendaal, The Netherlands) was used for cellular irradiation in the kilovoltage energy range. The beam qualities correspond to 50 kVp with additional filtration of 1.65 mm Al (HVL=1.58 mm Al), 125 kVp with additional filtration of 0.1 mm Cu and 2.5 mm Al (HVL=6.64 mm Al) and 250 kVp with additional filtration of 0.3 mm Sn, 0.5 mm Cu and 1.5 mm Al (HVL=2.4 mm Cu). Flasks were placed vertically 50 cm from the source with cell monolayers facing a 6x8  $\text{cm}^2$  applicator. The Axesse Elekta linear accelerator (LINAC) with beam modulator (Elekta AB, Kungstensgatan, Stockholm, Sweden) was used for cellular irradiation in the megavoltage energy range. Flasks were placed vertically at a depth of 2.2 cm and 1.5 cm in solid water to match the  $D_{\text{max}}$  depth of the 10 MV and 6 MV photon field, respectively. Solid water was placed both behind and around the sides of the flasks, primarily to provide adequate photon backscattering. All doses (1, 2, 3, 5 and 8 Gy) were delivered in a single fraction at room temperature. Unirradiated control samples (with and without drugs) were handled under the same conditions as the irradiated samples.

### 2.4. Clonogenic survival

Cell survival was measured by the colony-forming assay (Puck *et al.* 1956). Cells were plated after irradiation at low densities to achieve approximately 100 colonies after fifteen doubling times in 100-mm tissue culture dishes containing 10 mL of complete cell medium. Colonies were fixed and stained with a mixture solution of 25% crystal violet and 75% ethanol. Only colonies containing more than 50 cells were counted as surviving.

The surviving fraction (SF) expressed as a function of dose (D) curves were fitted using IgorPro software using the linear quadratic (LQ) model  $SF(D) = \exp(-\alpha D - \beta D^2)$  where  $\alpha$  [ $\text{Gy}^{-1}$ ] and  $\beta$  [ $\text{Gy}^{-2}$ ] are the radiosensitivity parameters (Chadwick *et al.* 1973; Curtis 1986; Fertil *et al.* 1981; 1985).

### 2.5. Sensitization enhancement Ratio

To quantify the increase in efficiency of treatment modality compared to non-treated control, we used  $SER_{10\%}$  or the ratio of the doses necessary to obtain the same radiobiological effect, i.e 10% cell survival.

### 2.6. Cell cycle analysis

Measurements of the cell cycle distribution were performed using flow cytometric analysis with propidium iodide (PI) staining. The method used was derived from Vine *et al.* (2007). Cells (2.0 x

10<sup>6</sup>) were treated with a solution containing 40 µg/mL PI, 100 µg/mL RNase A, and PBS (pH 7.4) at 37°C for 1 hour before the cell cycle analysis.

### 2.7. Immunocytochemistry

Cells incorporating BrUdR after drug treatment were detected using fluorescently labelled anti-BrUdR antibodies. The anti-BrdU mouse monoclonal antibody (Clone MoBU-1) conjugated to Alexa Fluor 488 (highly specific to BrUdR incorporated into the DNA) from Molecular Probe (Invitrogen, AU) was used. The assay was carried out using an acid denaturation method as per manufacturer's references with minor modifications.

### 2.8. Fluorescence measurements

The stained nuclei were analysed for both DNA-PI and Alexa Fluor-PI fluorescence using a Becton Dickinson fluorescence-activated cell sorting (FACS) flow cytometer (BD LSR II; BD Biosciences, USA) with excitation at 488 nm. The resulting DNA distribution was analysed using FlowJo software (V9.6.2; Tree Star Inc., USA).

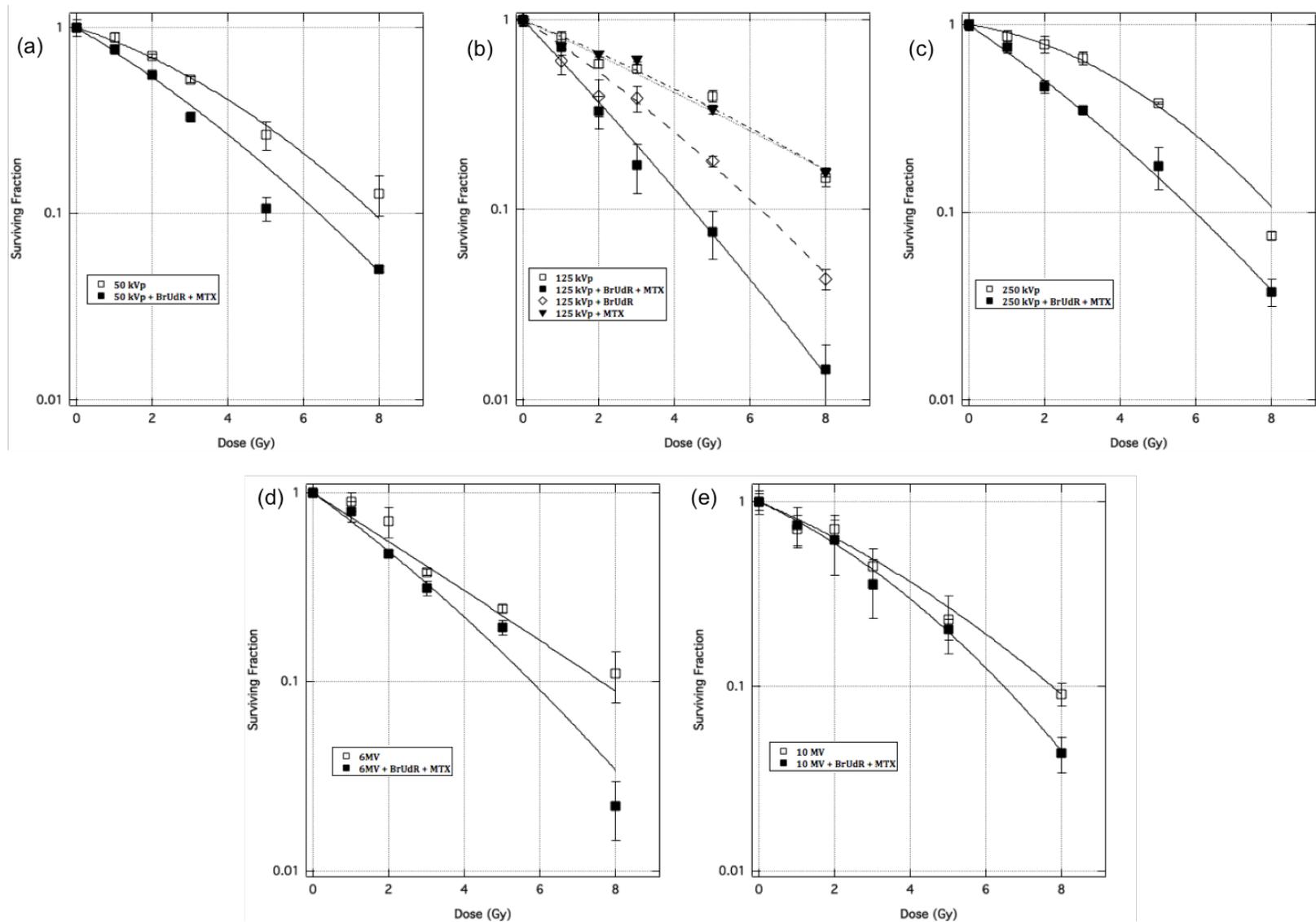
### 2.8. Statistical Analysis

Statistical analysis of toxicity data was performed using a two-tailed Student's t-test using the assumption of equal variance (p-value ≤0.05 considered significant).

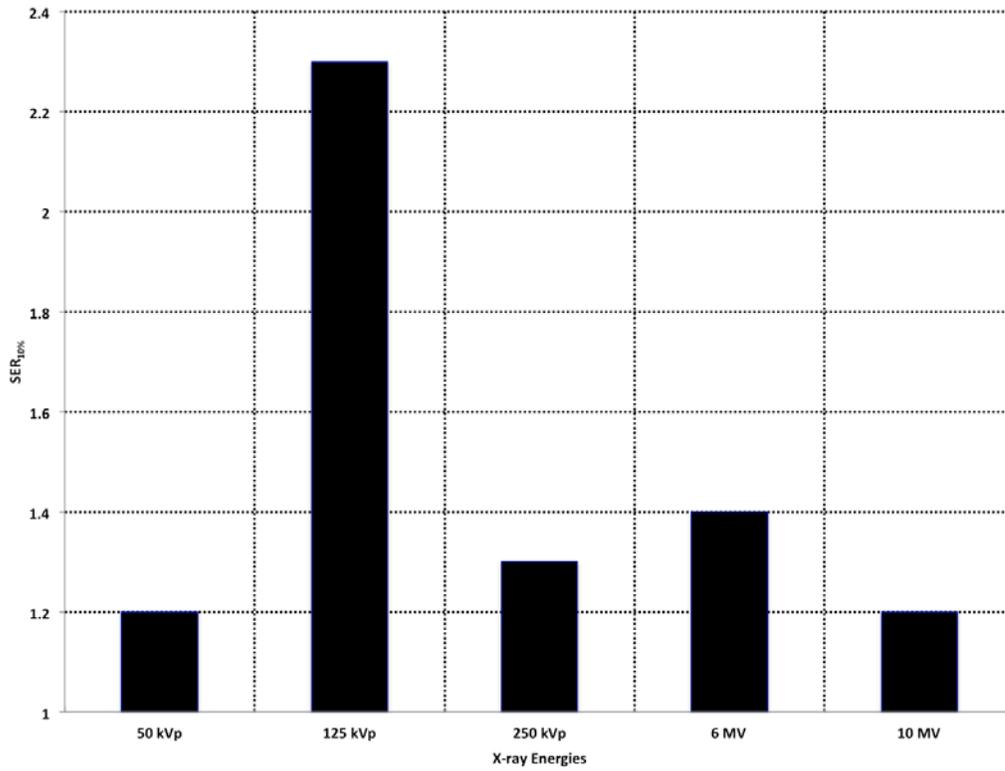
## 3. Results

### 3.1. Energy dependence of the radiation sensitization enhancement

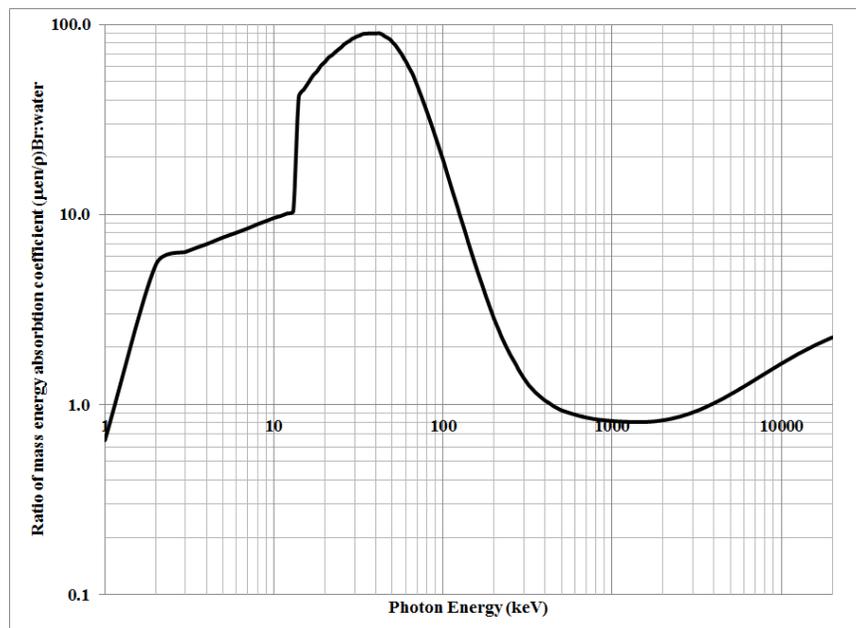
The survival curves show that at all energies, combining drugs (0.01 µM MTX and 10 µM BrUdR) with radiation is more effective than radiation alone (Figure 1a-1e). The enhancement exhibits an energy dependency and the maximum effect is observed at 125 kVp with a SER<sub>10%</sub> of 2.3 (Figures 1 and 2, Table 1). Figure 3 shows the dose gain produced by the replacement of water by bromine, as calculated by the ratio of the total mass energy absorption of bromine to water relative to the incident photon energy:  $(\mu_{en}/\rho)_{bromine}/(\mu_{en}/\rho)_{water}$ . The maximum value is at 40 keV, which is comparable to the effective energy of the spectrum emitted from the orthovoltage X-ray unit at 125 kVp.



**Figure 1.** Cell survival curves of 9L cells after irradiation at energies of 50 kVp (a), 125 kVp (b), 250 kVp (c), 6 MV (d), and 10 MV (e) with or without drugs (0.01  $\mu$ M MTX, 10  $\mu$ M BrUdR and the combination (0.01  $\mu$ M MTX and 10  $\mu$ M BrUdR)). Surviving fractions of irradiated cells only (no drugs) and with drugs were normalized to non-irradiated control and non-irradiated drugs-treated cells sample, respectively. Each data point represents the means  $\pm$  SD of at least two independent experiments (each triplicate).



**Figure 2.** Sensitization enhancement ratio at 10% surviving fraction (SER<sub>10%</sub>) of combined drugs (0.01 μM MTX and 10 μM BrUdR) vs. radiation energies.



**Figure 3.** Ratio of the mass energy absorption coefficient of bromine to water vs. photon energy (keV).

### 3.2. Efficacy of the different treatments at the optimum energy 125 kVp

Figure 1b shows clonogenic cell survival results for different pharmacological treatments, the corresponding radiobiological parameters ( $\alpha$ ,  $\beta$ ) are presented in table 1. In the presence of MTX the 9L cells have a similar radiosensitivity as the untreated cells (Figure 1b). This radiosensitivity increases in the presence of BrUdR with an additional effect when BrUdR is combined with MTX, confirming the radiation enhancement ability of the drug combination; a statistically significant difference between the groups was found ( $P < 0.003$ ). The 9L cells treated with the drug combination exhibit a linear trend in the survival curve (Figure 1b, Table 1). We believe that a synergistic effect appears as an increase in the  $SER_{10\%}$  as a result of the interaction with the BrUdR combined with the additional decrease of the DNA repair generated by MTX. The overall combined effect is supra-additive, i.e. greater than the sum of the individual components summed together.

**Table 1.** Values of the linear-quadratic model parameters  $\alpha$  and  $\beta$  and  $SER_{10\%}$  from 9L cells treated with ionising radiation only and BrUdR/MTX-sensitized radiation dose survival curves of different energies.

Energies	Treatment	$\alpha$ ( $Gy^{-1}$ ) <sup>a</sup>	$\beta$ ( $Gy^{-2}$ ) <sup>a</sup>	$SER_{10\%}$ <sup>b</sup>
50kVp	XIR <sup>c</sup>	$0.151 \pm 0.019$	$0.018 \pm 0.006$	1.0
	DRUGS <sup>d</sup>	$0.284 \pm 0.009$	$0.011 \pm 0.001$	1.2
125kVp	XIR <sup>c</sup>	$0.213 \pm 0.008$	$0.002 \pm 0.001$	1.0
	MTX alone	$0.192 \pm 0.013$	$0.004 \pm 0.002$	1.0
	BrUdR alone	$0.295 \pm 0.035$	$0.011 \pm 0.005$	1.6
	DRUGS <sup>d</sup>	$0.521 \pm 0.028$	N/A <sup>e</sup>	2.3
250kVp	XIR <sup>c</sup>	N/A <sup>e</sup>	$0.038 \pm 0.000$	1.0
	DRUGS <sup>d</sup>	$0.319 \pm 0.025$	$0.011 \pm 0.004$	1.3
6MV	XIR <sup>c</sup>	$0.200 \pm 0.015$	$0.010 \pm 0.002$	1.0
	DRUGS <sup>d</sup>	$0.335 \pm 0.024$	$0.011 \pm 0.005$	1.4
10MV	XIR <sup>c</sup>	$0.200 \pm 0.062$	$0.013 \pm 0.008$	1.0
	DRUGS <sup>d</sup>	$0.218 \pm 0.069$	$0.021 \pm 0.010$	1.2

<sup>a</sup> $\alpha$  and  $\beta$  are linear-quadratic model parameters. Values are presented as mean  $\pm$  SD.

<sup>b</sup> $SER_{10\%}$  = sensitization enhancement ratio at 10% surviving fraction.

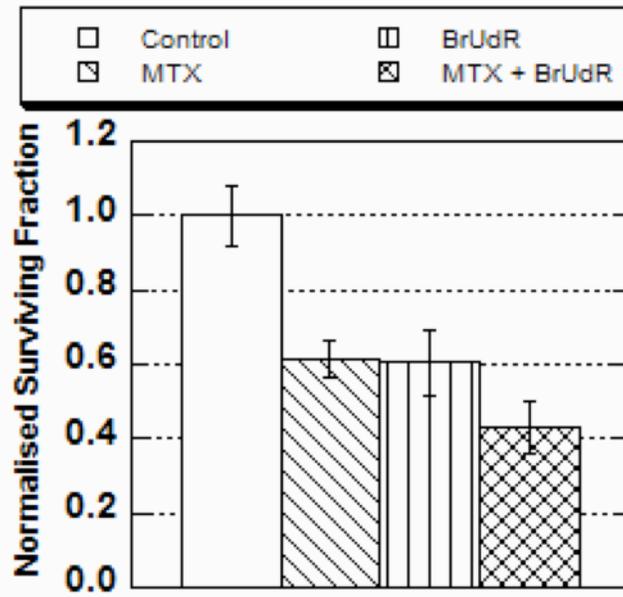
<sup>c</sup>XIR = X-ray radiation only.

<sup>d</sup>DRUGS = MTX (0.01  $\mu$ M) and BrUdR (10  $\mu$ M) combination.

<sup>e</sup>N/A = not applicable.

### 3.3. Drug cytotoxicity

Figure 4 shows that the 0.01  $\mu$ M MTX and/or 10  $\mu$ M BrUdR drug treatment of 9L cells induces toxic effects on the clonogenic survival. Surviving fractions for the MTX and the BrUdR treatments alone are similar with values of  $0.61 \pm 0.05$  (MTX vs untreated) and  $0.60 \pm 0.09$  (BrUdR vs untreated), respectively. The toxicity of the combined treatment is increased with a surviving fraction value of  $0.43 \pm 0.07$ .

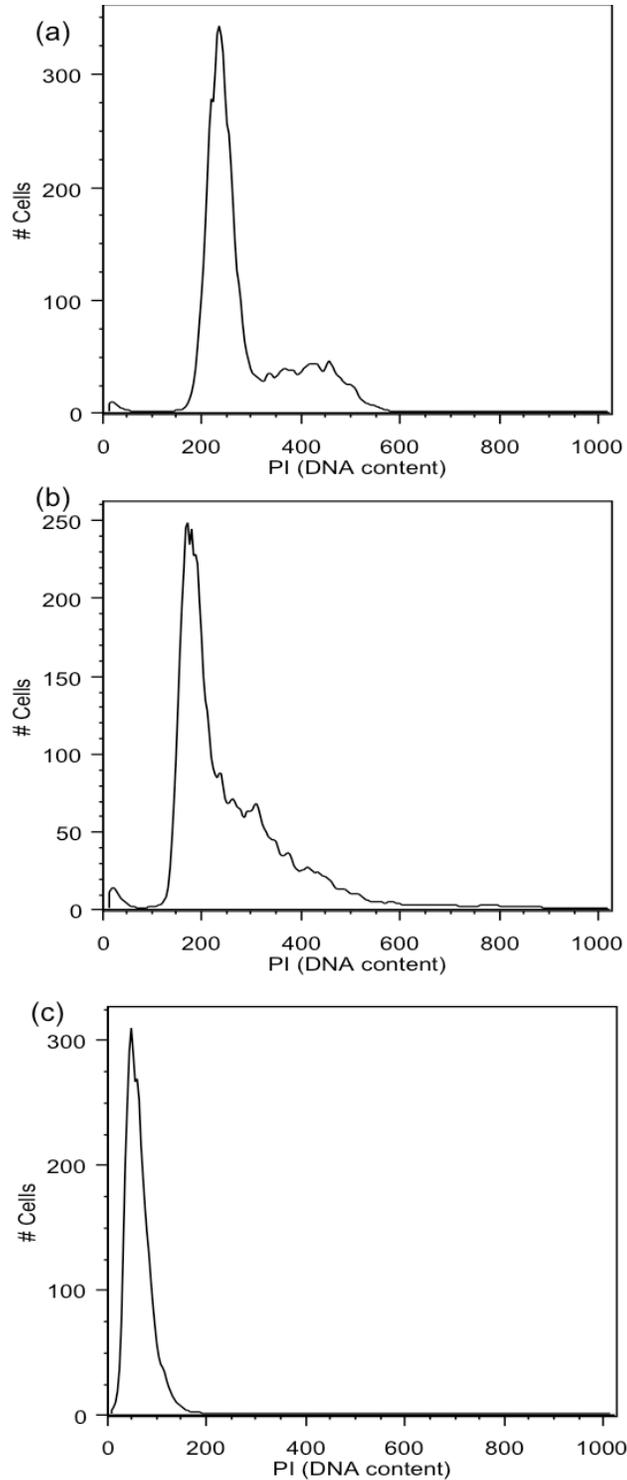


**Figure 4.** Cytotoxicity of the pharmacological treatments: 0.01  $\mu\text{M}$  MTX, 10  $\mu\text{M}$  BrUdR and the combination (0.01  $\mu\text{M}$  MTX and 10  $\mu\text{M}$  BrUdR) without radiation. Clonogenic surviving fractions were normalized to the untreated control. Bars are  $\pm$  one standard deviation ( $\pm\text{SD}$ ).  $p < 0.05$ .

### 3.4. Cell cycle analysis and bromine incorporation into DNA

Figure 5 shows a representative DNA histogram of PI stained 9L cells. The histogram profile of untreated control cells is shown in Figure 5a. Following drug incubation there is a decrease of cells in the G2/M phase associated with an accumulation into the S-phase (Figure 5b). For the drugs combined with radiation, an accumulation of the DNA in the pre-apoptotic sub-G1 peak is observed. The results show that the other cell cycle phases completely disappear (Figure 5c).

The incorporation of BrUdR in DNA was quantified using immunocytochemical staining. The cells were also counterstained with PI for DNA quantification. Alexa Fluor 488 fluorescent cells are considered BrUdR-positive cells. The BrUdR labelling index (LI) is the ratio of the number of BrUdR-positive cells to the total number of cells. The LIs of the cells treated with BrUdR and the combination MTX/BrUdR are similar, with values of  $38.4\% \pm 3.4\%$  and  $38.4\% \pm 3.1\%$  respectively.



**Figure 5.** Single parametric DNA diagrams of 9L cells stained with PI. Measurements were performed on a FACS LSR II flow cytometer without drugs and irradiation (a), with drugs alone (0.01  $\mu\text{M}$  MTX and 10  $\mu\text{M}$  BrUdR) (b), drugs (0.01  $\mu\text{M}$  MTX and 10  $\mu\text{M}$  BrUdR) combined with 5 Gy X-rays radiation of 125 kVp (c). FACS plots are representative of at least three independent experiments.

#### 4. Discussion

The experimental optimum energy for the maximum enhancement of BrUdR+MTX is at 125 kVp. This provides the highest  $SER_{10\%}$  of 2.3 (Figures 1 and 2, Table 1). The 125 kVp beam produces an effective energy of 46 keV which lies close to the energy predicted to maximize the dose enhancement (i.e. 40 keV) using BrUdR (Figure 3). Our results confirm the theoretical expectation (Figure 3) according to the maximum energy deposited in bromine compared to water and are thus in agreement with the formalism described by the authors (Corde *et al.* 2004). Our results also suggest that the production of photoelectrons and the resulting high LET Auger electrons have an important role in the enhancement energy dependence. Two earlier studies investigated the dose enhancement induced by halogenated pyrimidines on cells (Corde *et al.* 2004; Franken *et al.* 1997). Using human head and neck squamous carcinoma SQ20B and 50 keV monochromatic synchrotron beam (Corde *et al.* 2004) probed the dose enhancement induced by 10  $\mu$ M IUdR and found  $SER_{10\%} = 2.62$ . The significance of our work is that we were able to obtain similar  $SER_{10\%}$  results using a widely accessible X-ray treatment modality (orthovoltage machine as opposed to a synchrotron facility) with a considerably lower beam flux (compared to that at a synchrotron) and a much broader X-ray photon spectrum (i.e. not ideally optimized compared to the monoenergetic X-ray beam available at a synchrotron). Furthermore, we obtained comparable  $SER_{10\%}$  values using drugs with a lower atomic number (bromine compared to iodine) and we should therefore expect a higher  $SER_{10\%}$  using our treatment with IUdR.

If megavoltage beams would be in principle clinically more appropriate to treat deep-seated tumours, the MV treatments (Table 1) are not as effective as the treatment at 125 kVp, which was expected by the theoretical calculation (Figure 3). Based on the latter, we should however observe much lower  $SER_{10\%}$  values in this MV range. Other studies using cisplatin (Rousseau *et al.* 2010) and carboplatin (Bobyk *et al.* 2012) demonstrated an equivalent therapeutic response when the drug is combined with either 6 MV photons or synchrotron X-rays at the K-edge of platinum (78.8 keV). Our calculation for the element platinum suggests however that the optimum X-ray energy for the dose enhancement is also at about 40 keV and not at 78.8 keV. In a realistic clinical treatment scenario, kilovoltage beams could lead to appropriate dose distribution depending on patient size and localization of tumours. However beam energy optimization should not only take into account high-Z element but also overall irradiation geometry. Therefore the practical optimal beam energy might be ultimately higher than predicted in simple geometry.

To enrich our understanding of the radiobiological mechanism that may occur during our treatments, we study their effects at the dose enhancement optimal X-ray energy i.e 125 kVp, only more deeply. Several degrees of thymidine replacement by BrUdR have been observed on different cell lines (Delihis *et al.* 1962; Nagashima *et al.* 1985). Our result (38%) is reasonably close to the value (49%) reported on 9L at a similar BrUdR concentration (Nagashima and Hoshino 1985). Although MTX is toxic for 9L cells (Figure 4), it does not increase their radiosensitivity (Figure 1b) as they are not exponentially growing, being confluent in plateau phase (Spittle 1978). This observation is also a consequence of the MTX concentration used, which was 200-fold below the half maximal inhibitory concentration (IC<sub>50</sub>).

Individually MTX and BrUdR provoked similar toxicities on the 9L cells (Figure 4). However, while irradiation of BrUdR treated cells showed a significant decrease in cell survival there was no such decrease using MTX (Figure 1b). The significant decrease observed with BrUdR is related to its high-Z properties, as suggested above, and therefore its ability to act as a radiation

enhancer. Our results are consistent with previously published data (Larson *et al.* 1989). It has been suggested that the underlying mechanisms behind the increased radiation sensitivity induced by halogenated pyrimidines is due to an increase in the direct lethal DNA damages and/or a decrease of repair of sublethal damages (Franken *et al.* 2013; Franken *et al.* 1997). Both the  $\alpha$  and  $\beta$  parameters increased for the BrUdR treatment. The latter may indicate that there are increases of direct lethal DNA damages and accumulation of sublethal damages as suggested in (Franken *et al.* 2013; Franken *et al.* 1997). Addition of MTX exacerbates DNA damage repair impairment with linearization of the survival curve.

Data from the LQ model indicates an increased linearity of the survival curve in the presence of the drug combination (Figure 1b). The LQ model parameters indicate an increased radiosensitivity when the drug combination is present (Table 1). The increased linearization is indicative of a change in the local radiation spectrum. This observation suggests that more direct damage effects have occurred. This damage could be due to an enhancement of the lethal low energy electron field (resulting from the internalization of the high-Z drug component), which may be further magnified by a decrease in DNA repair (instigated by the presence of the thymidine analogue). The corresponding survival curve, obtained under the same irradiation conditions, for BrUdR is not linear with a lower  $\alpha$  value (Figure 1b, Table 1). We conclude that the increased linearity, with a higher  $\alpha$  value, is due to a synergetic combination of direct lethal DNA damage with a marked decrease in the DNA repair modulated by the MTX initiating apoptosis as suggested by the flow cytometric analysis (Figure 5C).

Pre-clinical evaluation of our radiation induced chemo-beta therapy in animal models is an important step toward potential clinical application. The complexity of physiological factors *in vivo* can largely affect the drugs delivery in tumours and lead to different tumour response to the treatment. To validate the effects observed *in vitro* on 9L gliosarcoma cell line, the model can be translated directly *in vivo* by using the Fischer 344 rat brain tumour model (Weizsaecker *et al.* 1981). Recently the blood-brain barrier disruption (Angelov *et al.* 2009) and nasal chemotherapy (Shingaki *et al.* 2010) have been explored as an alternative for administering anticancer drugs such as MTX for the treatment of brain tumours. Shingaki *et al.* (2010) investigated intranasal delivery of MTX to brain tumours (9L) and demonstrated significant direct transport of MTX to the brain. A reasonable approach for future *in vivo* experiments would be to use those techniques to deliver effective MTX-based chemotherapy and BrUdR concomitantly with precisely tuned external beam radiation therapy.

## 5. Conclusions

We have demonstrated the efficacy of radiation induced chemo-beta therapy. Our innovative multimodal approach for radio resistant brain tumour treatment uses the synergy of pharmacological treatments, an antimetabolite chemotherapy drug, MTX, and the halogenated pyrimidine, BrUdR, together with widely accessible hospital X-ray radiations. This study emphasizes the importance of linking drug activation to precise targeting of optimized energy X-ray beams leading to a highly effective chemo-radiation therapy. This is achievable with a significant decrease in the required drug and radiation dosage otherwise required for the same treatment outcome, which, if able to be translated into clinical practice, could potentially limit the toxicity of such a treatment to surrounding healthy tissues.

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