Synchrotron-based Photon Activation Therapy Effect on Cisplatin Pre-treated Human Glioma Stem Cells

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Abstract. *Background: Glioblastoma multiforme (GBM) is one of the deadliest cancers characterized by very limited sensitivity to chemo- and/or radiotherapy. The presence of GBM stem-like cells in the tumor might be relevant for GBM treatment resistance. Aim: To provide a proof-of-concept of the efficacy of photon activation therapy (PAT) using monochromatic synchrotron radiation (SR), in killing GBM stem cells pre-treated with cisplatin. Materials and Methods: Irradiation was performed using a 1-8 Gy dose range and energies just above or below the platinum K-shell edge (78.39 keV) or with a conventional X-ray source. Cells were exposed to drug concentrations allowing 90% cell survival, mimicking the unfavourable tissue distribution generally achieved in GMB patients. Results: a significant enhancement in cell lethality was observed using SR compared to conventional X-ray irradiation. Conclusion: PAT deserved to be further explored in in vivo models based on GBM stem-like cells.*

Glioblastoma multiforme (GBM) is a malignant cancer with a well-established, extraordinary resistance to conventional radiotherapy and chemotherapy, usually resulting in a very rapid, unfavourable disease course despite aggressive combined therapy. Among the possible reasons for GBM treatment resistance, the difficulty in achieving effective concentrations in the tumour of otherwise highly effective antineoplastic drugs (1, 2) and the presence of glioma stem cells (GSCs) able to survive to radiation and chemotherapy, thus renewing the cancer cells killed by treatment (3, 4), have been identified. Therefore, to overcome the limitation of available treatments, original approaches are mandatory to enhance the effectiveness of the present therapies.

Photon activation therapy (PAT) is one of these new approaches to treat highly-resistant cancers, based on the possibility to exploit in radiotherapy the high Z-number of some metal-based drugs, such as platinum, gold or silver. Using kiloVolt X-rays, the large photoelectric cross-section of the heavy-Z elements results in substantially increased interaction with X-rays, producing primary and secondary low-energy electrons that can produce DNA double strand breaks (5). Interestingly, the short range of these electrons can produce a localized dose enhancement in the tumour volume without damaging the surrounding normal tissues.

The efficacy of using X-rays to irradiate tumors previously loaded with drugs containing heavy metals is well-documented (5-8) and this has been also demonstrated in *in vivo* GBM models (9, 10). Due to the presence of platinum in its molecule, cisplatin (CDDP) is potentially photoactivable at the platinum K-edge energy (78.4 keV) using synchrotron radiation (SR). For this purpose high-energy X-ray beams with sufficient fluence, such as the ones available at the European Synchrotron Radiation Facility (ESRF) and used for this study, are required.

In the present study we tested the survival of GBM2 GSCs line to different doses of radiation either delivered using a conventional X-ray generator or monochromatic X-rays at energies bracketing the K-edge of platinum. The results of these assays will provide the scientific rationale for developing *in vivo* protocols using PAT and targeting cancer stem cells.

Materials and Methods

Cell lines. GBM2 GSCs were originally isolated from surgery-derived GBM WHO grade IV tumor specimens from a male patient, as described elsewhere (11). The GBM2 cell line used in

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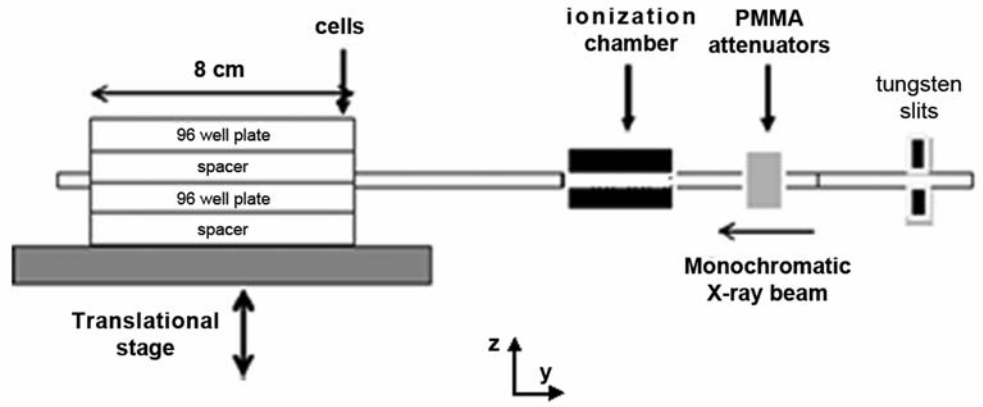

Figure 1. *Set-up of synchrotron irradiation of biological samples performed at the ID17 biomedical beamline of the ESRF. X-rays are propagating from right to left. The monochromatic X-ray beam is shaped by a set of tungsten slits. PMMA attenuators of 0-24 cm thickness can be chosen at will to modify the X-ray intensity without changing the spectrum. The entrance dose is on-line monitored by an ionization chamber. Ninety six-well plates are placed in on a vertically movable stage. Only the first line is filled with cells. Drawing not to scale.*

the present study was kindly provided by Dr. A. Daga (National Institute for Cancer Research, Department of Hematology-Oncology, Genova, Italy). The stemness properties of GBM2 cell line was monitored during this study in order to ensure that the data obtained could actually be ascribed to the stem cell subpopulation of GBM. Along the study period, GBM2 cells retained a good proliferation rate, ability to form neurospheres and did not enter the differentiation program, as shown by the stable expression of stem cell markers (CD133 and nestin). Furthermore a complete genomic and epigenomic profile has been drawn (12). Cells were grown in Dulbecco's Modified Eagle's medium (DMEM)/F-12 and Neurobasal Medium (1:1) supplemented with B27 supplement minus Vitamin A (1:50, Life Technologies, Paisley, UK), recombinant human fibroblast growth factor 2 (10 μg/ml, Milteny Biotech, Bergisch Gladbach, Germany), recombinant human epidermal growth factor (10 μg/ml, Milteny Biotech). Under these conditions, the cells attach and grow as a monolayer in flasks and maintain an intact self-renewal capacity for at least 3 months.

SR irradiation. The irradiation was performed at the ESRF biomedical beamline (ID17). At the ESRF, the X-ray source is a 21-pole wiggler that produces a continuous and intense X-ray spectrum up to 400 keV. By using a silicon 111 monochromator system (13), quasi-monochromatic X-ray beams can be selected in the energy range 25-200 keV. Two photon energies were used for this experiment: 77.4 keV and 79.4 keV (energy spread of the beam: 0.4 keV), bracketing the platinum K-absorption edge found at 78.39 keV. Regardless of the energy selected, the beam was fixed in the horizontal plane; irradiations were performed by vertically scanning the sample in front of the beam.

Cells were irradiated in sets of four stacked multiwell-plates at room temperature in aerobic conditions (Figure 1).

Along the x directions, cell-containing plates were placed only in the centre of the plate in order to ensure that they received the same dose of radiation. Furthermore, along the y direction, cells were plated only in the first row (upstream with respect to the beam propagation) of the 96-well plate in order to avoid differences in irradiation doses due to the possible attenuation of the beam through the different wells.

The beam was horizontally homogeneous (inhomogenity <7.5%) over its 4.0 cm lateral (x direction in Figure 1) width, sufficient to cover the cell-containing plates. In the vertical direction, the SR beam was naturally highly collimated; in our case it was defined by a set of tungsten slits to a height of 1.10 mm. The dose was monitored on-line using a ionization chamber (Oken Ltd, Kumagawa, Japan), placed upstream of the cells and previously calibrated against a PTW 31010 chamber connected to a PTW electrometer (PTW, Freiburg, Germany) (Figure 1). By changing the magnetic field of the wiggler X-ray source or by varying the thickness of the polymethylmethacrylate (PMMA) absorbers in the monochromatic X-ray beam upstream the samples, the dose rate could be varied of 1-2 orders of magnitude without changing the beam energy. The number of scans necessary to deliver the predefined dose depended on the chosen dose rate and on the beam height. In this study the dose rate was approximately 30 cGy per scan, corresponding to a vertical translation at 2.5 mm/s. Plates were irradiated with a dose range of 1-8 Gy.

After irradiation, cells were incubated for 6 days. The incubation period was chosen on the basis of the cell doubling time in order to avoid an underestimation of radiation-induced cell death and cell survival was determined by the Sulforhodamine B (SRB) assay (see below).

Conventional irradiation. In order to compare the effect of the combined CDDP and radiotherapy treatment, GBM2 GSCs irradiation was also performed with a conventional X-ray clinical irradiator RADGIL (Gilardoni, Milano, Italy). The dose rate was 0.468 Gy/min at 190 kVp, 12 mAs, in a dose range of 1-8 Gy. The spectrum has been calculated using the SpecCalc program (14).

Combination of SR/conventional irradiation with CDDP treatment. Cells were plated in 96-well plates, as previously described, and treated with CDDP (Sigma-Aldrich, St Louis, MO, USA). A drug stock solution of 1 mg/ml was freshly-prepared every time in sterile saline solution and diluted to the final concentration in culture

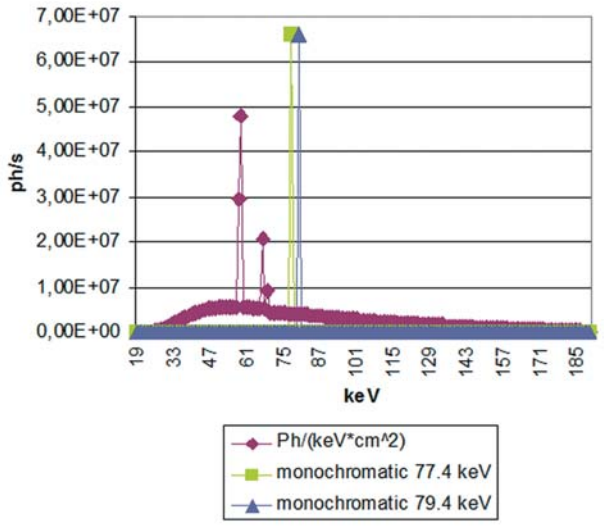

Figure 2. *Emission spectrum of the conventional X-ray source, calculated for the 190-kVp, minimum energy 19 keV, beam filtration value 0.3 mm Cu, 0.5 mm Be, emission angle 37 degrees, bin 1 keV. The synchrotron monochromatic lines at 77.4 and 79.4 keV are also reported. Intensity for synchrotron radiation is in arbitrary units.*

medium just before use. GBM2 GSCs were treated with CDDP 0.5 and 1 μM. CDDP concentrations were selected based on preliminary studies in order to achieve a low cytotoxicity (*i.e.* lower than 10%), thus allowing to simulate the unfavorable condition frequently achieved in the core of GBM in affected patients and the effect of combined CDDP+SR or conventional irradiation to be easily detected.

After 24 h of treatment the drug containing medium was removed and replaced with fresh medium just before cell irradiation. Untreated cells and cells treated only with CDDP or radiation were used as controls.

SRB assay. Cells were harvested with trypsin-EDTA when they were in exponential growth, counted and plated in 96-well plates. The optimal seeding density was chosen to ensure exponential growth during the assay and 500/well GBM2 GSCs were plated. In order to evaluate the effects of the selected treatments, the culture medium was aspirated and cells were fixed with 10% cold trichloroacetic acid for 1 hour at 4°C. Cells were then washed with deionised water and stained with 0.1% SRB (Sigma Aldrich, St Louis, MO, USA) dissolved in 1% acetic acid for 15 minutes at room temperature. After incubation, cells were washed with 1% acetic acid to remove the excess of stain and plates were air-dried at room temperature. Bound protein stain was then solubilised with 10 mM tris(hydroymethyl)aminomethane and the optical density (OD) at 540 nm was determined using a microplate reader (Victor II, PerkinElmer, Waltham, MA, USA) (8, 15).

Data analysis. The survival rate was calculated as (mean OD treated cells/mean OD control cells)x100. Data were expressed as mean value±SD and analyzed by ANOVA followed by Tukey's multiple comparison test with the GraphPad Prism (GraphPad Software, San Diego, CA, USA) statistical package, setting the level of significance at $p<0.05$.

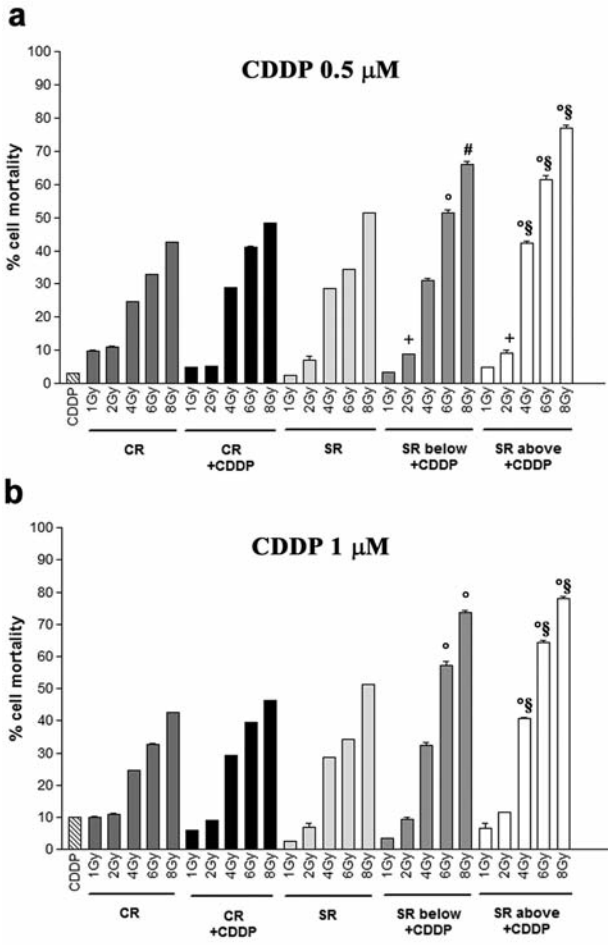

Figure 3. *Cell mortality (expressed as % with respect to untreated control cells) of GBM2 GSCs treated with cisplatin 0.5 μM (a) or 1 μM (b), conventional or synchrotron X-rays alone or chemo-radio combination treatment. Since no significant differences in survival were found between irradiations above or below the platinum K-edge (78.4) keV, only results above the platinum K-edge are reported. Results are expressed as mean±standard deviation of at least 3 replicates (One-way ANOVA, Tukey's post test: °p<0.001 vs. conventional radiation plus cisplatin and SR alone; #p<0.05 vs. conventional radiation plus cisplatin and SR alone; +p<0.01 vs. conventional radiation plus cisplatin; §p<0.001 vs. synchrotron radiation below platinum K-edge plus cisplatin). CDDP, cisplatin; CR, conventional radiation; SR, synchrotron radiation.*

Results

In Figure 2 we report on the filtered spectrum of the conventional irradiator compared with the synchrotron radiation monochromatic energies used in this study, according to the results obtained with the SpecCalc program (14).

Non-irradiated cells were incubated with CDDP 0.5 or 1 μM allowing a cell survival of 97 and 90% respectively with respect to untreated control cells.

Figure 3 shows the cell mortality (with respect to untreated control cells) induced by the different treatments: CDDP (0.5 or 1 μM); conventional radiation; monochromatic synchrotron X-rays above the platinum K-edge (78.4 keV); CDDP-plus-conventional radiation; CDDP plus monochromatic synchrotron X-rays below or above the platinum K-edge (78.4 keV).

Both types of X-ray irradiation alone induced a dose-dependent cell death in the selected dose range, with a maximum lethality around 50% at 8 Gy in both cases. A significant difference ($p<0.01$) *vs.* CDDP treatment alone was observed starting from 1 Gy and 4 Gy, respectively, when compared to CDDP 0.5 or 1 μM, and no significant difference could be evidenced between synchrotron and conventional irradiation used alone (Figure 3).

When the effect of X-ray irradiation was assessed on cells previously exposed to CDDP, a significant effect of SR irradiation above or below the platinum K-edge *vs.* SR-alone was evident almost along the entire dose range of irradiation used in our experimental paradigm. In fact, starting from a dose of 2 Gy and up to 8 Gy, SR induced a significant enhancement in cell lethality in cells pre-treated for 24 h despite their exposure to very low doses of CDDP with respect to cells incubated in CDDP-free medium.

The increase in GBM2 GSCs mortality induced by SR in combination with CDDP was significantly higher with respect to the one observed for conventional radiation plus CDDP starting from 2 or 4 Gy dose when cells were pre-treated with CDDP 0.5 or 1 μM, respectively (Figure 3).

Furthermore, irradiation of GBM2 GSCs with synchrotron X-rays above the platinum K-edge (78.4 keV) pre-treated with CDDP at both the selected concentrations induced a slight, but significant ($p<0.001$), increase in cell mortality in 4-8 Gy dose range with respect to that observed following an irradiation below the edge (Figure 3).

Discussion

PAT has gained increasing interest in view of its theoretical capacity to remarkably enhance cytotoxicity in cancer cells with limited local toxicity on normal tissues, particularly when obtained using SR. In the field of experimental neuro-oncology, very promising results have been obtained with SR-PAT coupled with CDDP pre-treatment both *in vitro* and *in vivo* glioma models (5-17), as well as in non-glioma *in vitro* models (8). However, the efficacy of combined CDDP+SR treatment has not yet been tested on the stem-like counterpart of GBM that are suggested to be major determinants of GBM resistance to treatment, *i.e.* GSCs.

Our study, aimed at providing evidence for a possible effectiveness of combined CDDP+SR treatment in GSCs, we also reproduced the frequent, and highly unfavorable, event represented by the difficulty of achieving effective concentrations of the anti-neoplastic drug in the tumor. On this regard, we selected CDDP concentrations for GBM2 GSCs pre-treatment allowing a cell survival rate equal or higher to 90%.

We assessed the treatment-induced cell mortality with the simpler SRB test and not with the clonogenic assay, supported by our previous results in similar experimental conditions (8) and by the results of other studies where it was used for chemo- and radio-sensitivity testing (15).

Optimal cell seeding concentrations and incubation periods have been selected in order to correctly estimate the radiosensitivity of these cells, confirming previously reported data (8, 15, 18).

Regarding the selection of the cellular model to be used in the study, GBM2 GSCs have been extensively characterized for their stem cell properties (sphere-forming assays, evaluation of differentiation properties, marker expression, *in vivo* engraftment). Their complete insensitivity to epidermal growth factor receptor (EGFR) kinase inhibitors (erlotinib and gefitinib) has been reported elsewhere (19), as well as their cytogenomic and epigenomic profiles (11, 12).

Based on our previous experiments (8) and on literature data (5, 9), SR treatments were performed around the platinum K-edge. In fact, the X-ray absorption of platinum is greatly increased at an energy level just above the K-edge (78.39 keV) and the maximal DNA damage induction is observed at around 80 keV when the photoactivable drug is localized inside the cells (9). Furthermore, low energies (<40 keV) were not used because of their low prospects for use in future human treatments.

This proof-of-concept *in vitro* study confirms and allows to extend to GSCs the promising results already reported in the rat glioma model (5), thus corroborating the interest of this therapeutic approach in poor-prognosis GBL treatment. In fact, SR proved to be significantly superior to conventional X-ray treatment on CDDP pre-treated cells at the same dose delivered.

Phase I clinical trials on metastatic brain tumors is ongoing at ESRF to test the safety and efficacy of the SR protocol (20). The potential clinical relevance of our experimental results is further boosted by the likely availability in the next few years of smaller, compact and cheaper devices, called Thomson sources, able to produce quasi-monochromatic radiation. These sources would allow disseminating the technique outside the large-scale facilities like synchrotrons (see for example http://equipex-thomx.fr/) (21).

In conclusion, our results show a significant increase in cell lethality in GSCs induced by SR-PAT in CDDP pre-treated cells with respect to the effect observed with conventional irradiation for the same dose and provide further support to the suggestion that this innovative therapeutic strategy may represent a novel approach to GBM treatment, thus deserving to be further explored in relevant *in vivo* models.

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References

1 Groothuis DR: The blood-brain and blood-tumor barriers: a review of strategies for increasing drug delivery. Neuro-Oncology *2*: 45-59, 2002.

2 Huynh GH, Deen DF and Szoka FC Jr: Barriers to carrier mediated drug and gene delivery to brain tumors. J Control Release *110*: 236-259, 2006.

3 Reya T, Morrison SJ, Clarke MF and Weissman IL: Stem cells, cancer, and cancer stem cells. Nature *414*: 105-111, 2001.

4 Nduom EK, Hadjipanayis CG and Van Meir EG: Glioblastoma cancer stem-like cells: implications for pathogenesis and treatment. Cancer J *18*: 100-106, 2012.

5 Biston MC, Joubert A, Charvet AM, Balosso J and Foray N: *In vitro* and *in vivo* optimization of an anti-glioma modality based on synchrotron X-ray photoactivation of platinated drugs. Radiat Res *172*: 348-358, 2009.

6 Corde S, Balosso J, Elleaume H Renier M, Joubert A, Biston MC, Adam JF, Charvet AM, Brochard T, Le Bas JF, Estève F and Foray N: Synchrotron photoactivation of cisplatin elicits an extra number of DNA brakes that stimulate RAD51-mediated repair pathways. Cancer Res *63*: 3221-3227. 2003.

7 Corde S, Joubert A, Adam JF, Charvet AM, Le Bas JF, Estève F, Elleaume H and Balosso J: Synchrotron radiation-based experimental determination of the optimal energy for cell radiotoxicity enhancement following photoelectric effect on stable iodinated compounds. Br J Cancer *91*: 544-551, 2004.

8 Ceresa C, Nicolini G, Requardt H, Le Duc G, Cavaletti G and Bravin A: Photon activation therapy's effect on cisplatin pre-treated human tumour cell lines: comparison with conventional X-ray irradiation. Journal of Biological Regulators & Homeostatic Agents *27*: 477-485, 2013.

9 Bencokova Z, Balosso J and Foray N: Radiobiological features of the anti-cancer strategies involving synchrotron X-rays. J Synchrotron Radiat *15*: 74-85, 2008.

10 Corde S, Biston MC, Elleaume H, Estève F, Charvet AM, Joubert A, Ducros V, Bohic S, Simionovici A, Brochard T, Nemoz C, Renier M, Troprès I, Fiedler S, Bravin A Thomlinson W, Le Bas JF and Balosso J: Lack of cell death enhancement after irradiation with monochromatic synchrotron X rays at the K-shell edge of platinum incorporated in living SQ20B human cells as cis-diamminedichloroplatinum (II). Radiat Res *158*: 763-770, 2002.

11 Griffero F, Daga A, Marubbi D, Capra MC, Melotti A, Pattarozzi A, Gatti M, Bajetto A, Porcile C, Barbieri F, Favoni RE, Lo Casto M, Zona G, Spaziante R, Florio T and Corte G: Different response of human glioma tumor-initiating cells to epidermal growth factor receptor kinase inhibitors. J Biol Chem *284*: 7138-7148, 2009.

12 Baronchelli S, Bentivegna A, Redaelli S, Riva G, Butta V, Paoletta L, Isimbaldi G, Miozzo M, Tabano S, Daga A, Marubbi D, Cattaneo M, Biunno I and Dalprà L: Delineating the cytogenomic and epigenomic landscapes of glioma stem cell lines. PLoS One 8:e57462. doi: 10.1371/journal.pone.0057462, 2013.

13 Suortti P, Fiedler S, Bravin A, Brochard T, Charvet AM, Elleaume H, Schulze-Briese C and Thompson AC: Fixed-exit monochromator for computed tomography with synchrotron radiation at energies 18 to 90 keV. J Synch Radiat *7*: 340-347, 2000.

14 Poludniowski G, Landry G, DeBlois F, Evans PM and Verhaegen F: SpekCalc: a program to calculate photon spectra from tungsten anode x-ray tubes. Phys Med Biol *7*: 54: N433-438, 2009.

15 Pauwels B, Korst AE, de Pooter CM, Pattyn GG, Lambrechts HA, Baay MF, Lardon F and Vermorken JB: Comparison of the sulforhodamine B assay and the clonogenic assay for *in vitro* chemoradiation studies. Cancer Chemother Pharmacol *51*: 221-226, 2003.

16 Biston MC, Joubert A, Adam JF, Elleaume H, Bohic S, Charvet AM, Estève F, Foray N and Balosso J: Cure of Fisher rats bearing radioresistant F98 glioma treated with cis-platinum and irradiated with monochromatic synchrotron X-rays. Cancer Res *64*: 2317-2323, 2004.

17 Ceresa C, Bravin A, Cavaletti G, Pellei M and Santini C: The Combined Therapeutical Effect of Metal-based Drugs and Radiation Therapy: The Present Status of Research. Current Medicinal Chemistry *21*: 2237-2265, 2014.

18 Carmichael J, DeGraff WG, Gazdar AF, Minna JD and Mitchell JB: Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res *47*: 936-942, 1987.

19 Ropolo M, Daga A, Griffero F, Foresta M, Casartelli G, Zunino A, Poggi A, Cappelli E, Zona G, Spaziante R, Corte G and Frosina G: Comparative analysis of DNA repair in stem and nonstem glioma cell cultures. Mol Cancer Res *7*: 383-392, 2009.

20 Estève F, Adam JF, Balosso J, Berkvens F, Bravin A, Elleaume H, Le Bas JF, Nemoz C, Tessier A and Renier M: Monochromatic radiotherapy: From first experimental glioma bearing rat therapy up to the first clinical trial. European Journal of Cancer 49: supplement *2*: S8-S8, 2013.

21 www.ClinicalTrials.gov, identifier: NCT01640509 o.

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