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Polo-like kinase 1 inhibitor BI6727 sensitizes 9L gliosarcoma cells to ionizing irradiation

Nan Li
University of Wollongong, nli@uow.edu.au

Elette Engels
University of Wollongong, ee215@uowmail.edu.au

Jeremy A. Davis
University of Wollongong, jeremyd@uow.edu.au

Andrew Dipuglia
University of Wollongong, ad150@uowmail.edu.au

Sarah Vogel
University of Wollongong, sv855@uowmail.edu.au

See next page for additional authors

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Polo-like kinase 1 inhibitor BI6727 sensitizes 9L gliosarcoma cells to ionizing irradiation

Abstract

Surgery, chemotherapy and radiotherapy remain as the major treatment strategies for cancers. Some agents such as anti-cancer drugs have capacity to enhance the radiation sensitivity of cancer cells at G2/M phase, leading to an improved radiotherapeutic efficacy. BI6727 is an ATP-competitive pololike kinase 1 (Plk 1)inhibitor and an anti-cancer drug. Using the radio-resistant 9L rat gliosarcoma cells as model, we examined the effect of BI6727 on cell growth and assessed the chemoradiotherapeutic efficiency between 150 kVp conventional irradiation (dose rate of 0.76 Gy min−1) and 66 keV synchrotron x-ray broad beam irradiation (dose rate of 46 Gy s−1). Our studies showed that BI6727 significantly caused cell growth arrest at G2/M phase and inhibited 9L cell proliferation with EC50 of 58.1 nM. In combinatory treatment, irradiation of BI6727-treated 9L cells with synchrotron x-rays at a dose rate of 46 Gy s−1 resulted in significant reduction of the cell survival compared to the conventional x-rays at a dose rate of 0.76 Gy min−1. These results indicated that Plk1 inhibitor BI6727 enhanced radio-sensitization of 9L cells in a dose rate dependent manner. For clinical application, irradiation with high dose rate is a promising strategy to improve chemo-radiotherapeutic efficacy for gliosarcoma cancer.

Keywords

gliosarcoma, 9l, sensitizes, bi6727, inhibitor, 1, irradiation, kinase, ionizing, polo-like, cells

Disciplines

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Authors

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Polo-like kinase 1 inhibitor BI6727 sensitizes 9L gliosarcoma cells to ionizing irradiation

Nan Li[1,2], Elette Engels[1,2], Jeremy A. Davis[1,2], Andrew Dipuglia[1], Sarah Vogel[1,2], Michael Valceski[1,2], Anatoly B. Rosenfeld[1], Michael L. F. Lerch[1,2], Stéphanie Corde[1,2,3] and Moeava Tehei[1,2]

1 Centre for Medical Radiation Physics, Wollongong, NSW, Australia

2. Illawarra Health and Medical Research Institute, Wollongong, NSW, Australia

3. Radiation Oncology Department, Prince of Wales Hospital, Randwick, NSW, Australia

Corresponding Author: Moeava Tehei, email: moeava@uow.edu.au

Key words: Plk1, BI6727, G2/M arrest, DNA damage, radio-sensitization

Abstract

Surgery, chemotherapy and radiotherapy remain as the major treatment strategies for cancers. Some agents such as anti-cancer drugs have capacity to enhance the radiation sensitivity of cancer cells at G2/M phase, leading to an improved radiotherapeutic efficacy. BI6727 is an ATP-competitive polo-like kinase 1 (Plk 1) inhibitor and an anti-cancer drug. Using the radio-resistant 9L rat gliosarcoma cells as model, we examined the effect of BI6727 on cell growth and assessed the chemo-radiotherapeutic efficiency between 150 kVp conventional irradiation (dose rate of 0.76 Gy/min) and 66 keV synchrotron X-ray broad beam irradiation (dose rate of 46 Gy/sec). Our studies showed that BI6727 significantly caused cell growth arrest at G2/M phase and inhibited 9L cell proliferation with EC_{50} of 58.1 nM. In combinatory treatment, irradiation of BI6727-treated 9L cells with synchrotron X-rays at a dose rate of 46 Gy/sec resulted in significant reduction of the cell survival compared to the conventional X-rays at a dose rate of 0.76 Gy/min. These results indicated that Plk1 inhibitor BI6727 enhanced radio-sensitization of 9L cells in a dose rate dependent manner. For clinical application, irradiation with high dose rate is a promising strategy to improve chemo-radiotherapeutic efficacy for gliosarcoma cancer.

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1. Introduction

Brain cancer remains as one of the most aggressive incurable cancer types. Gliosarcoma (GSM) is a very rare neoplasm with an incidence of 1-8% of all malignant gliomas [1]. GSM has similar epidemiology and natural history to glioblastoma multiforme (GBM), the most common primary brain cancer with a median survival of about 15 months [1-2]. GSM and GBM usually recur despite maximum treatment with less than 3% to 5% of survival rate over five years [3]. The overall survival is typically 3 months without any treatment [4]. Treatment for brain cancer involves surgery, chemotherapy and radiation therapy. However, radio-resistance is common in GBM and GSM [2]. Hence, developing methods by involving novel anti-cancer drugs with radio-sensitization potential and new radiotherapy modalities would be the path to largely improve the radiotherapeutic efficacy.

The anti-cancer drug volasertib (BI6727) is a polo-like kinase 1 (Plk1) inhibitor [5-6]. Plk1 plays critical roles in eukaryotic cell division by regulating centrosome maturation, spindle assembly, microtubule–kinetochore attachment and cytokinesis during mitosis [7-8]. Plk1 overexpression in cancer cells correlates with prognosis of cancer progression [9]. Inhibition of Plk1 in cancer cells causes G2/M phase arrest, mitotic catastrophe and apoptosis [10-11]. It was reported that Plk1 inhibitors inhibited cancer cell growth including brain cancer cells and increased their radio-sensitivity [12-18]. Significant improvement of local tumour control was observed in an *in vivo* study with combinatory treatment of Plk1 inhibition and fractionated irradiation [19].

X-ray dose rate is an important factor that affects biological consequences such as cell survival for a given absorbed dose. It was reported that low dose rate with extended exposure time would increase cell survival comparing to high dose rate [20]. Therefore, dose rate is a crucial factor in radiotherapy.

In this work, radio-resistant 9L gliosarcoma cell line was used as a model to investigate the combinatory effect of BI6727 with low dose-rate conventional and high dose-rate synchrotron broad beam irradiation. The 9L cell line is the most widely used experimental rat brain tumor model to study the treatment strategies for aggressive

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brain tumours [21]. Here we reported a newly developed chemo-radiotherapy that significantly increased the mortality of radio-resistance brain cancer cells.

2. Methods

2.1. Cell culture

Rat 9L gliosarcoma cell line was obtained from the European Collection of Cell Cultures (ECACC). Cells were cultured in DMEM with L-Glutamine and high glucose (Gibco, Life Technologies) supplemented with 10% fetal bovine serum (Bovogen Biologicals) and 1% penicillin/streptomycin (10,000 units/ml) (Gibco, Life Technologies). Cell growth was maintained in 37 °C humidified atmosphere with 95% air and 5% CO2.

2.2. Drug treatment with Plk1 inhibitor BI6727

Plk1 inhibitor BI6727 (Selleckchem) was reconstituted in dimethyl sulfoxide (DMSO) according to the manufacturer's instructions and stored at -80 ^{o}C. BI6727 was added to cell culture with the final DMSO concentration of 0.1% in the culture. Cells treated with DMSO alone were used as vector-treated control.

2.3. Measurement of cell growth by MTT proliferation assay

9L cells (2000 cells/well) were seeded to 96-well plates and allowed to attach and grow at 37 ^{o}C for 24 hours prior to BI6727 treatment. Cells were then treated with different concentrations of BI6727 for 72 hours. Cell proliferation was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay using Vybrant® MTT Cell Proliferation Assay Kit (Molecular Probes). The plates were read using a SpectraMax Plus 384 Microplate Reader at wavelength of 540 nm. Experiments were carried out in triplicate and background absorbance was subtracted from all wells before analysis.

2.4. Cell cycle analysis by flow cytometry

9L cells were incubated with BI6727 at 50 nM for 24 hours before being detached from T25 flasks (Corning). The collected cells were fixed with 70% ethanol and stained with propidium iodide (PI). The stained cells were then analyzed using a Becton Dickinson

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fluorescence-activated cell sorting (FACS) flow cytometer (BD LSR II; BD Biosciences, USA). The data was analysed using BD FACSDiva Software.

2.5. Combination of BI 6727 and ionizing radiation

9L cells were cultured in Falcon 12.5 cm² cell culture flasks (T12.5 flasks). Cells were incubated with 50 nM of BI6727 for 24 hours prior to irradiation. A radiation dose of 8 Gy was delivered as a single fraction from conventional and synchrotron X-ray sources, respectively.

Conventional irradiation on 9L cells at low dose rate was performed in the Radiation Oncology Department at the Prince of Wales Hospital (POWH) (Randwick, NSW, Australia) with a Nucletron Oldelft Therapax DXT 300 Series 3 Orthovoltage unit (Nucletron B.V., Veenendaal, The Netherlands). The tube potential was 150 kVp with additional filtration of 0.35 mm Cu and 1.5 mm Al, resulting in a beam of 0.67 mm Cu half value layer, with a dose rate of 0.76 Gy/min at 50 cm from the source. The irradiation set-up and the photon energy spectrum were described elsewhere [22]. Briefly, monolayer confluent cells in flasks were delivered 8 Gy at 6 mm-depth in medium in a horizontal orientation with solid water placed around the sides and beneath the flasks to maintain a full scatter condition.

Irradiation on 9L cells at high dose rate was conducted at hutch 2B on Imaging and Medical Beam Line (IMBL) in the Australian Synchrotron (AS). The flasks with 9L cells were filled with Hank's Balanced Salt Solution (HBSS) (Gibco, Life Technologies). Each flask was irradiated vertically in a specifically designed solid water phantom, the cell monolayers being 2.5 cm downstream. The pink broad beam was produced by 2.2 T wiggler field and filtered by Cu-Al filters (without ex-vacuo) with mean weighted energy of 66 keV and dose rate of 46 Gy/sec. This beam energy was chosen to match as closely as possible the effective energy of the conventional 150 kVp X-ray source [23]. Therefore, the energy effect would be negligible when comparing the radiation effects on cell survival from the two X-ray sources.

2.6. Clonogenic assay

9L cells were grown in T12.5 flasks for the treatments with/without BI6727 and/or irradiation. After the treatment, cells in each flask were re-seeded to Corning Primaria

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100 mm cell culture dishes as described previously [24]. After 15 doubling times, the plates were washed and stained with crystal violet solution. Only colonies with at least 50 cells were counted as surviving and normalized to the untreated control to calculate the surviving fractions [24].

2.7. Immunofluorescence detection of γH2AX by flow cytometry

To stop DNA repair during the conventional irradiation in POWH, 9L cells in T12.5 flasks were irradiated on ice. The cells were then incubated at 37 ^{o}C for 1 hour before being collected for the assay. H2AX phosphorylation assay kit for flow cytometry (Upstate Biotechnology) was used for the detection of cellular γH2AX in response to DNA damage with a modified protocol [25]. The FITC-labeled cells were counter stained with 10 µg /ml PI. The FITC and PI double labeled cells were analyzed using a Becton Dickinson fluorescence-activated cell sorting (FACS) flow cytometer (BD LSR II; BD Biosciences, USA). The data was analyzed using BD FACSDiva Software.

2.8. Immunofluorescence detection of γH2AX by fluorescence microscopy

For immunofluorescence imaging, 50 nM of BI6727 was applied to 9L cells grown in T12.5 flasks for 24 hours, followed by conventional irradiation in POWH or synchrotron irradiation in AS, as stated in 2.5. For conventional irradiation, 9L cells in flasks were irradiated on ice and were incubated for 20 min (37°C 5 min and room temperature 15 min) before the cell fixation was performed. While for the synchrotron irradiation, the flasks were irradiated at room temperature and then were kept at room temperature for 20 min before the cell fixation step. Synchrotron irradiation took less than one minute, the DNA repair during irradiation was neglected.

The immunofluorescence staining procedures were performed using the method described previously [26]. Mouse anti-γH2AX (Ser139) antibody (Upstate Biotechnology, 1:500 dilution) and goat anti-mouse antibody conjugated with Alexa-488 (Molecular Probes, 1:500 dilution) were applied. γH2AX images were acquired at room temperature with a Leica DMI4000B inverted fluorescence microscope with a 10x objective lens.

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The γH2AX fluorescence intensity in the images was analyzed using program ImageJ [27] and a modified method for counting particles (https://microscopy.duke.edu/guides/count-nuclear-foci-ImageJ). In brief, the nucleus with γH2AX fluorescence were counted as particles (the white color spots in images). Therefore, the number of particles could represent the number of cells with γH2AX signals. The 'analyze particles' program available in ImageJ was used to count the number of particles after an intensity threshold was set (from 0 to 65535 for the 16 bit images used). The initial intensity threshold was set low to count all of the particles which represented all of the cells in the image (the total particles Nt). By gradually increasing the intensity threshold, the number of counted particles (or cells) were recorded as they changed as a function of γH2AX fluorescence intensity. The proportion of counted particles which related to the cell population at each threshold was normalized as a percentage of the total particle number Nt. The correlation between the cell population and γH2AX intensity could be obtained by plotting this serial intensity thresholds to the cell populations. The γH2AX intensity in non-irradiated cells was considered as background, which was subtracted from the plot for respective irradiated cell images.

2.9. Statistical analysis

Statistical significance was calculated using Student's unpaired two-tailed *t*-test. The level of statistical significance was chosen as $P < 0.05$. Error bars represent the standard deviation (SD) of the mean ($n \geq 3$). GraphPad Prism 7 was used to calculate EC_{50} values and to compute the nonlinear regression equations.

3. Results

3.1. Plk 1 inhibitor BI6727 suppressed 9L cell growth by inducing G2/M arrest

To assess the inhibition of 9L cell growth by BI6727, MTT assay was carried out by incubating 9L cells for 72 hours with different concentrations of BI6727 in a range of 1 nM to 10 μM. As shown in Figure 1, BI6727 significantly inhibited the proliferation of 9L cells with an EC_{50} of 58.1 nM (Figure 1a).

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Strong G2/M phase arrest was also observed in cells treated with BI6727 at 50 nM for 24 hours. Cell cycle analysis showed an increased cell population of 51.6% at G2/M phases when compared to the vector-treated control (26.7%, figure 1b). Given that cells in G2/M phase are more susceptible to irradiation than in other phases [28], accumulation of cells in G2/M phase induced by BI6727 would increase the sensitivity of cells to applied ionizing irradiation.

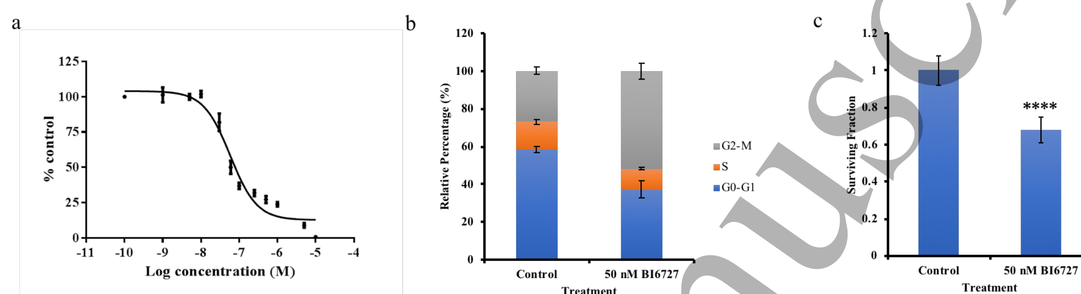

Figure 1. Plk1 suppressed the proliferation and colony formation of 9L cells by inducing mitotic arrest. a. MTT based proliferation assay. 9L cells were incubated with different concentrations of BI6727 for 72 hours. EC_{50} was calculated to be 58.1 nM using GraphPad. b. BI6727 induced G2/M arrest in 9L cells. 9L cells were incubated with 50 nM BI6727 for 24 hours and the cell cycle was analyzed using PI staining and flow cytometry. c. Clonogenic assay to assess the intrinsic cytotoxicity of a 24 hours exposure of 9L cells to 50 nM BI6727. DMSO-treated cells were used as control. Data represents a mean ± SD from three independent experiments. Bars indicate the standard deviation (SD). ****$P \leq 0.0001$

3.2. Inhibition of Plk 1 reduced colony-forming ability of 9L cells

Clonogenic assay was used to assess the cell survival or division capability after drug treatment [29]. As indicated in Figure 1c, the survival of 9L cells was reduced significantly to 68.0% after 24 hours of treatment with 50 nM BI6727 when compared to the control ($P \leq 0.0001$) (Figure 1c).

3.3. Plk1 inhibitor BI6727 enhanced radio-sensitivity of 9L cells in a dose rate dependent manner

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It is well known that cells at G2/M phase are more sensitive to radiation [28]. To assess whether BI6727-induced G2/M arrest in 9L cells could enhance radio-sensitization as well as the impact of dose rate on the enhancement, irradiation was carried out using two X-ray sources with different dose rates. A dose of 8 Gy to the cells was delivered as a single fraction for both irradiations. As indicated in Fig. 2, irradiation with synchrotron X-ray source (AS) at a dose rate of 46 Gy/sec led to a high reduction of cell surviving fraction compared to the conventional X-ray source at a dose rate of 0.76 Gy/min (POWH). This occurred for both non-BI6727-treated control (11.2% vs. 16.0%; $P<0.001$, columns c and a) and BI6727-treated 9L cells (7.0% vs. 13.4%, $P<0.0001$, columns d and b). For the cells treated with the conventional X-ray source, combinatory treatment with BI6727 was able to decrease the surviving ability of 9L cells with slight significance (13.4% vs. 16.0%; $P<0.05$, Fig. 2, columns b and a). However, for the cells treated with synchrotron X-ray source, combinatory treatment with BI6727 significantly reduced the survival of 9L cells compared to the control with irradiation-only (7.0% vs. 11.2%, $P<0.001$. Fig. 2, columns d and c). These results indicated that BI6767 treatment was able to enhance the sensitivity of 9L cells to irradiation and the enhanced radio-sensitivity is likely dose rate dependent.

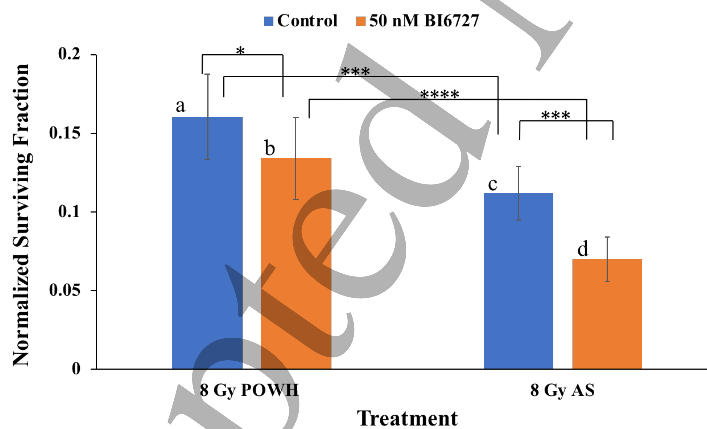

Figure 2. BI6727 sensitized 9L cells to irradiation. 9L cells were treated with 50 nM BI6727 for 24 hours prior to irradiation that was delivered to a dose of 8 Gy in a single fraction from two different X-ray sources: POWH (energy 150 kVp, dose rate of 0.76 Gy/min) and AS (energy 66 keV, dose rates of 46 Gy/sec). The cell survival was assessed by clonogenic assay. Column a: control with conventional irradiation only; column b: BI6727 in combination with conventional irradiation; columns c: control

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with synchrotron irradiation only; column d: BI6727 in combination with synchrotron irradiation. The surviving fractions were calculated by normalizing to non-irradiated vector-treated cells (for columns a and c) or non-irradiated BI6727-treated cells (for columns b and d), respectively. Data represents mean ± SD from three independent experiments. $^{*}P \leq 0.05$, $^{***}P \leq 0.001$ and $^{****}P \leq 0.0001$.

3.4. Combinatory treatment significantly induced DNA damage in G2/M arrested cells

Plk1 plays critical role in mitosis and maintaining the genome stability. Disruption of Plk1 function has a potential to cause DNA damage. Further, irradiation induces more DNA damage on G2/M arrested cells. To assess the accumulated effect of DNA damage induced by BI6727 and irradiation, we measured γH2AX signals in the non-treated and treated cells using flow cytometry and immunofluorescence imaging techniques.

Without BI6727 treatment, the cell populations were largely distributed in G0-G1 phases and the γH2AX signals induced by ionizing irradiation were mainly assigned to these populations (Figure 3a and 3b). BI6727 along can cause DNA damage with weak γH2AX intensity (Figure 3c). Upon exposing to irradiation, high γH2AX intensity (in purple color) was observed in G2/M phase cells indicating the formation of large amount of γH2AX foci in response to double-strand DNA breaks (Figure 3d).

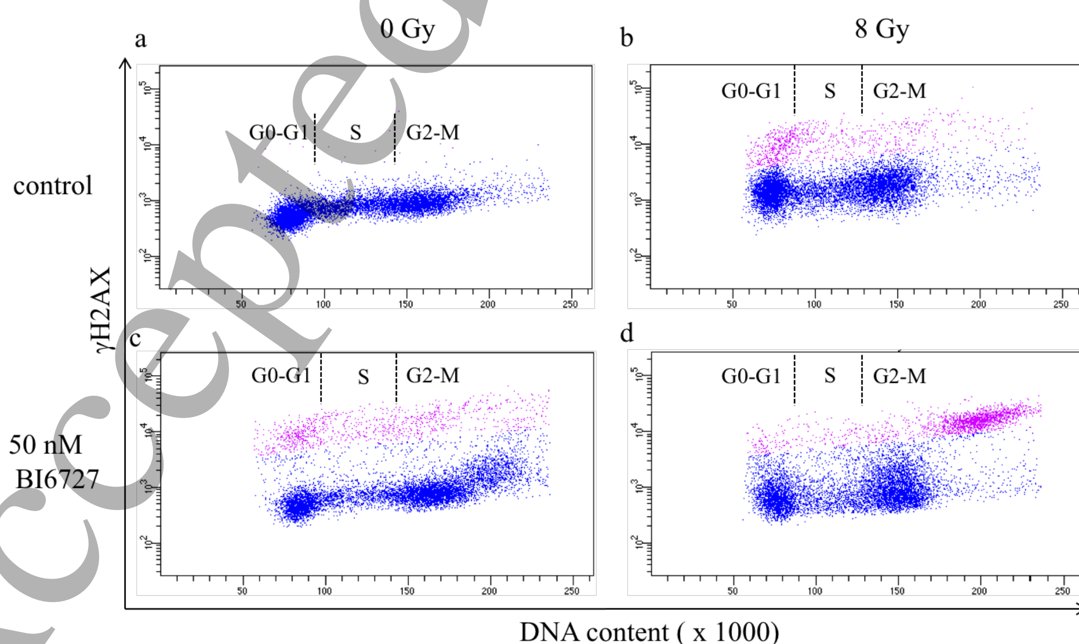

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Figure 3. Bivariate PI-combined γH2AX analysis by flow cytometry in 9L cells after the treatment of BI6727 and x-ray irradiation. (a) vector-treated control; (b) 8 Gy irradiation; (c) 50 nM BI6727 for 24 hours; (d) 50 nM BI6727 for 24 hours and 8 Gy irradiation. The 8 Gy irradiation was delivered in a single fraction from POWH 150 kVp X-ray source. The cells were stained with FITC conjugated anti-phospho-H2AX (Ser139) and PI. γH2AX fluorescence detected with different intensity was shown as blue (low intensity) and purple (high intensity). The experiment was repeated three times.

DNA damage from these treatments was further observed by immunofluorescence imaging analysis. As indicated in Figure 4a, more and/or stronger γH2AX foci were observed in cells exposed to the synchrotron irradiation comparing to the conventional irradiation treatment. Further, when analyzing the cell populations vs. γH2AX fluorescence intensity, higher γH2AX fluorescence intensity was observed in BI6727-treated cells exposed to either synchrotron or conventional irradiation (Figure 4b). After a threshold pixel intensity above 35000, only the γH2AX signals from BI6727-treated cells exposed to synchrotron irradiation remained. This indicated that the combinatory treatment of BI6727 and synchrotron irradiation induced higher DNA damage over the conventional irradiation in BI6727-treated cells.

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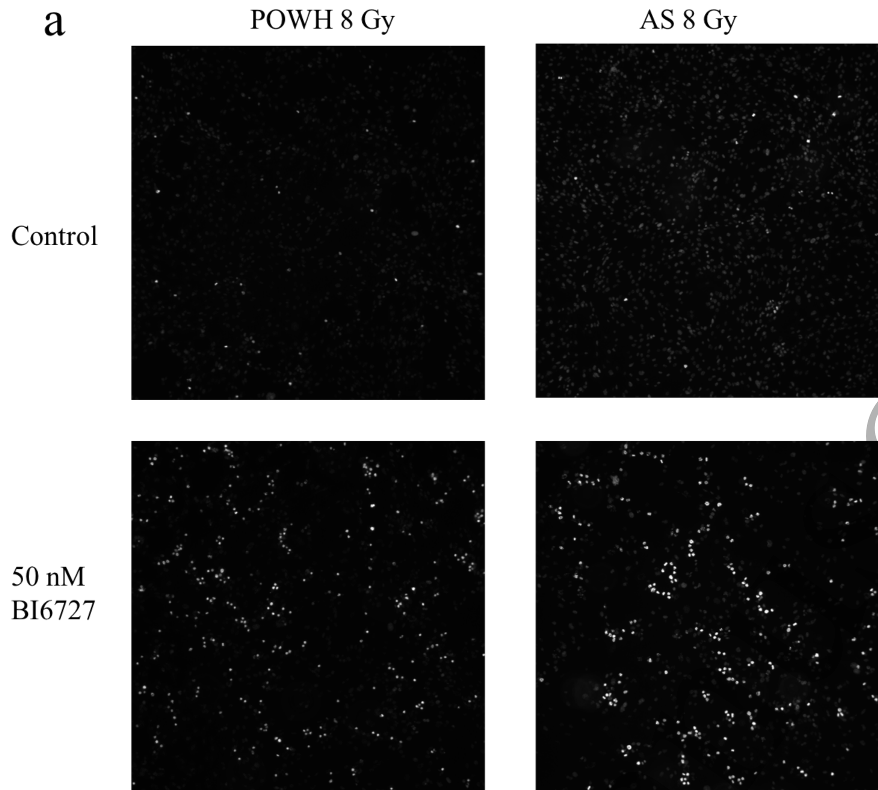

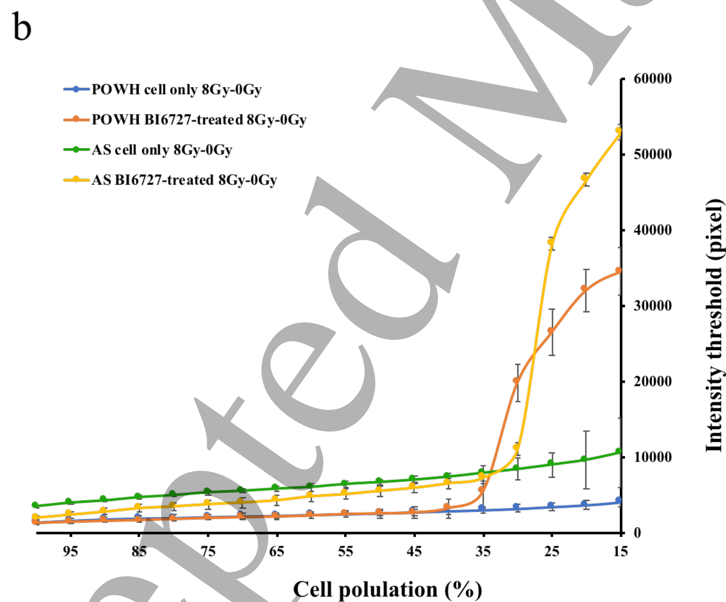

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Figure 4. Immunofluorescence imaging for γH2AX detection. 9L cells were treated with 50 nM BI6727 for 24 hours prior to 8 Gy irradiation from POWH 150 kVp and AS 66 keV X-ray sources, respectively. (a) Cellular γH2AX foci (in white) were detected by anti-γH2AX (Ser139) primary antibody and Alexa-488 conjugated secondary antibody. Images are representatives of three repeats. (b) The comparison of

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the plots between the cell population and γH2AX intensity for non-BI6727-treated and BI6727-treated 9L cells after exposing to conventional and synchrotron 8 Gy irradiation. The γH2AX fluorescence images were analyzed using ImageJ. Images were collected and analyzed from three repeats.

4. Discussion

Plk1 is a potential anti-cancer target due to its overexpression in many cancer cells but not in normal tissues. Many Plk1 inhibitors have been developed for cancer intervention with a few drugs being currently applied in clinical trials [5-6]. Of these, BI6727, an ATP competitive Plk1 inhibitors, inhibits cancer cells significantly at nanomolar concentrations and has better pharmacokinetics profile [6].

In our study we firstly characterized the inhibitory ability of BI6727 on 9L cells. The inhibitory effect of BI6727 on 9L is modest with EC_{50} of 58.1 nM when compared to other cell lines treated with BI6727 and similar ATP competitive Plk1 inhibitors BI2536 and GSK463416 [15, 30-32]. Cell survival measured by clonogenic assay reduced to 68.0% when treated with 50 nM BI6727 for 24 hours, which is comparable to the survival of most of the reported brain tumour cell lines treated with 50 nM BI2536 (the SFs were between 33.6% and 69.7%) [16]. Thus, BI6727 at 50 nM was used in the combinatory chemo-irradiation treatment throughout.

Plk1 inhibition induces G2/M arrest and enhances radio-sensitization in many cancer cells. In our study, we showed that the Plk1 inhibitor BI6727 remarkably induced cell arrest in G2/M phase and increased more sensitivity of the radio-resistant 9L cells to high dose-rate synchrotron irradiation than to the low dose-rate conventional irradiation. The reduced cell survival after ionizing irradiation is related to DNA damage. Indeed, we have observed enhanced stronger γH2AX signals in BI6727-treated cells exposed to synchrotron irradiation than those exposed to conventional irradiation, indicating the increased DNA damage in synchrotron irradiation.

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9L cell line is well known for its radio-resistance comparing with other rat brain tumour cells [33-34]. We previously reported that there was no survival change at given doses up to 8 Gy with two different dose rates of 0.5 Gy/min and 5 Gy/min from 10 MV irradiation [35]. However, the present study demonstrated a significant dose rate effect on the survival of 9L cells at the given dose of 8 Gy with the dose rate increased from 0.76 Gy/min to 46 Gy/sec. A similar dose rate effect on 9L cell survival was also observed in our previous study [36]. In this case, at the given dose of 2 Gy, the cell survival was reduced from 74% to 32% when the dose rate increased from 0.76 Gy/min to 50 Gy/sec from conventional and synchrotron broad beam irradiation respectively, although the beam energy for synchrotron irradiation was 137 keV [36]. Hence, the dose rate is critical for inhibitory effect of post-irradiation survival in 9L cells and the dose rate effect must be considered for the future *in vivo* studies when utilising high dose-rate irradiation.

Recent studies investigating FLASH radiotherapy have demonstrated that there is a significant dose rate dependence between healthy tissue and tumours [37-39]. High dose rates (>40 Gy/s) have been shown to increase normal tissue sparing [37] and maintain tumour control [38]. A recent animal study demonstrated that ultra-high dose rate synchrotron X-rays could also trigger the FLASH effect [40]. In this study, the normal brain injury in mice was prevented after a 10 Gy whole brain irradiation (mean dose rate 37 Gy/s) [40]. This has pointed out a promising future for our study when we extend our current work to preclinical studies using the synchrotron X-ray source.

There is a high demand on development of novel treatment strategies such as new anti-cancer drugs and new radiotherapy modalities. In addition to the high selectivity and inhibitory ability, BI6727 was reported to be able to pass through the blood brain barrier, making it a promising anti-brain tumour drug [18]. It is agreeable that more work is required on measuring the cell survival and the associated DNA damage and repair dynamics for different dose rate irradiation, our current findings provide evidence that combing PLK inhibitor with high dose rate irradiation could also become a new path in killing strong radio-resistant cancer cells

5. Conclusion

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Radio-resistance is a major problem for brain tumor radiotherapy. Developing new treatment strategy to reduce radio-resistance and thereof to improve therapeutic efficiency is beneficial for cancer patients. BI6727 treatment significantly improves the sensitivity of 9L cells to irradiation in a dose-rate dependent manner, suggesting an efficient treatment strategy for radio-resistant gliosarcoma cancer.

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