Radiosensitisation of glioblastoma cells using a histone deacetylase inhibitor (SAHA) comparing carbon ions with X-rays

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Abbreviations: Glioblastoma (GBM), O\textsuperscript{6}-methylguanine DNA methyltransferase (MGMT), Poly (ADP-ribose) polymerase (PARP), Suberoylanilidehydroxamic acid (SAHA), Temozolomide (TMZ), Linear energy transfer (LET), Histone Deacetylase (HDAC), Histone Deacetylase inhibitor (HDACi), Double Strand Breaks (DSB), Sensitiser Enhancement Ratio (SER), Relative Biological Effect (RBE), Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR), Sensitiser Enhancer Ratio (SER), Yttrium Aluminum Garnet doped with Cerium (YAG:Ce), Ataxia telangiectasia mutated (ATM)
Abstract

Purpose: Prognosis for patients with glioblastoma (GBM) remains poor, and new treatments are needed. Here we use a combination of two novel treatment modalities, carbon ions and a histone deacetylase inhibitor (HDACi). We compare these to conventional X-rays, measuring the increased effectiveness of carbon ions as well as radiosensitisation using HDACi.

Materials and methods: Suberoylanilide hydroxamic acid (SAHA) was used at a non-toxic concentration of 0.5 µM in combination with 85 keV µm\(^{-1}\) carbon ions, and 250 kVp X-rays for comparison. Effects were assayed using clonogenic survival, γH2AX foci repair kinetics and measuring chromatin decondensation.

Results: Dose toxicity curves show that human GBM LN18 cells were more sensitive to SAHA compared to U251 cells at higher doses, but there was little effect at low doses. When combined with radiation, clonogenic assays show that the Sensitiser Enhancement Ratio with carbon ions at 50% survival (SER\(_{50}\)) was about 1.2 and 1.5 for LN18 and U251 respectively, but was similar for X-rays at about 1.3. The repair half-life of γH2AX foci was slower for cells treated with SAHA and was most noticeable in U251 cells treated with carbon ions where after 24 hr, more than double the number of foci remained in comparison to the untreated cells. Hoechst fluorescent dye incorporation into the nucleus showed significant chromatin decondensation and density homogenisation with SAHA treatment for both cell lines.

Conclusions: Our results suggest a vital role of histone deacetylases (HDAC) in the modulation of DNA damage response and support the use of SAHA for the treatment of GBM through the combination with heavy ion therapy.
**Introduction**

Suberoylanilide hydroxamic acid (SAHA or Vorinostat) is an epigenetic agent that inhibits both class I and class II deacetylases (HDAC). SAHA’s mode of action is primarily through chromatin modification, as hyper-acetylation of histones upon HDAC inhibition leads to chromatin decondensation. This generally leads to an upregulation of gene transcription in transformed cell lines (Gui et al. 2004), influencing a wide range of cellular processes including proliferation, apoptosis and DNA damage response (Minucci and Pelicci 2006).

SAHA has been the first HDAC inhibitor (HDACi) to be approved by the United States Food and Drug Administration (FDA) for the treatment of relapsed and refractory cutaneous T-cell lymphoma (Dalgaard et al. 2008; Gui et al. 2004). There have been a limited number of Phase I and Phase II clinical trials using SAHA on patients with recurrent glioblastoma, either as a single agent or in combination with other drugs (Chinnaiyan et al. 2012; Friday et al. 2012; Galanis et al. 2009). The results obtained so far suggest that SAHA is well tolerated and tumour progression, in some cases, has been delayed.

SAHA has been shown to enhance radiosensitisation with conventional X-rays in a variety of cancer cell types both in vitro and in vivo with little or no toxicity to normal cells (Blattmann et al. 2010; Dalgaard et al. 2008). The mechanism of radiosensitisation is related to disrupted repair of DNA, leading to increased cell death (Groselj et al. 2013). It is known that SAHA can inhibit and down-regulate Rad51 which is crucial to DNA repair by homologous recombination (HR), while it also interferes with Ku70/Ku80 proteins involved in non-homologous end-joining (NHEJ) DNA repair. The consequence of this is the longevity of γH2AX foci (which signal regions of double strand breaks, DSB) in irradiated cells and their correlated radiosensitivity (Oertel et al. 2011; Blattmann et al. 2010). Interestingly, SAHA induces apoptosis in some cancer cells, but not in equivalent normal cells, when combined with either X-rays or carbon ions. It has been postulated that this unexpected finding is related to differing transcriptional regulation between cancer and normal cells (Qiu et al. 1999; Minucci and Pelicci 2006). In particular the cyclin-dependent kinase inhibitor 1 gene (p21WAF1), which in general prevents proliferation, is suppressed in many tumour cells but is upregulated by SAHA resulting in cell death (Gui et al. 2004).

Heavy ion therapy (HIT) is an alternative radio-therapeutic modality to X-rays, characterized by a greater radiobiological effect for the same dose, normal tissue sparing due to the high energy
deposition in the Bragg Peak, and an increased efficacy of killing hypoxic tumour cells. Glioblastoma (GBM) brain tumour is a good candidate for treatment with HIT, as lower doses and a more confined treatment volume could spare vital eloquent areas of the brain, while greater cell killing in hypoxic areas could lower recurrence. Patients with GBM are routinely treated with concomitant X-rays and temozolomide (TMZ), but prognosis still remains poor (Barazzuol et al. 2010). Previously, we have shown that TMZ causes reproducible additive cytotoxicity when combined with radiation, regardless of the radiation types and only on 0\textsuperscript{6}-methylguanine DNA methyltransferase (MGMT)-methylated GBM cell lines (Barazzuol et al. 2011), while ABT-888 (a poly (ADP-ribose) polymerase (PARP) inhibitor) radiosensitises all tested cell lines regardless of their MGMT status (Barazzuol et al. 2013). Despite advances in brain tumour treatment, it is clear that new therapies need to be developed to improve life expectancy. Importantly, it has been shown that SAHA can cross the blood-brain barrier and increase histone acetylation levels in the brain tissue (Hockly et al. 2003; Yin et al. 2007).

Relatively few studies have investigated the radiosensitising effect of HDACi combined with carbon ions (Kano et al. 2009; Oertel et al. 2011). It is proposed that a major mechanism of SAHA radiosensitisation is through disrupting the repair and signalling response to DNA DSB. As carbon ions predominantly induce complex DSB through direct interaction with DNA while X-rays predominantly produce single strand breaks (SSB) through indirect interaction (by free radical production), it is thought that the efficacy of combining carbon irradiation with SAHA should be even greater than with X-rays. In this paper, we compare the effects of SAHA combined with carbon ion and X-ray irradiation on two GBM cell lines (LN18 and U251).

**Methods**

*Cell lines and culture*

Human GBM U251 cells were obtained from the Health Protection Agency Culture Collections (HPACC, Salisbury, Wiltshire, UK) and Human GBM LN18 cells from the American Type Culture Collection (ATCC, Teddington, Middlesex, UK). The cells were cultured as previously described in Barazzuol *et al.* (2013). The doubling time for both cell lines was approximately 24 hours, whereas the plating efficiency was 63 ± 2\% for LN18 and 24 ± 2.6\% for U251.

*Drug Treatment*
SAHA (Sigma-Aldrich, Poole, Dorset, UK) was reconstituted in pure MilliQ water. For the toxicity assay (Figure 1) the cells were cultured at an appropriate concentration of drug continuously for about 2 weeks. For radiosensitisation assays (Figure 2), SAHA was diluted to a concentration of 0.5 µM in cell media and incubated with cells for 24 hr before the cells were irradiated, and removed directly after irradiation.

**Carbon ion irradiation**
Cells were irradiated using broad beam carbon ions provided by the Heavy Ion Medical Accelerator (HIMAC) at the National Institute of Radiological Sciences (NIRS), Japan, at an energy of 290 MeV/u and a Linear Energy Transfer (LET) of 85 keV µm⁻¹ on target, and a dose rate of 1Gy/min. The facility and broad beam irradiation procedure has been described elsewhere (Kanai et al. 1999; Torikoshi et al. 2007). Cells were irradiated in either T25 flasks or microscopy flasks (Nunc, New York, USA) at a confluence of about 80%.

**X-ray irradiation**
X-ray irradiation was performed using a Gulmay machine operating at 250 kVp with a dose rate of 0.65 Gy/min (Royal Surrey County Hospital, Guildford, UK).

**Clonogenic assay**
Clonogenic assays were used to evaluate the effect of SAHA with radiation. After irradiation, cells were seeded at an appropriate concentration for the dose received and grown in 6-well plates and incubated for up to 14 days. Colonies were fixed with 50% ethanol in Phosphate Buffered Saline (PBS) and then stained with 5% crystal violet in PBS (Sigma-Aldrich). Colonies with more than 50 cells were counted and the survival fractions determined by taking into consideration the plating efficiency for all treatment modalities based on three separate experiments.

**γH2AX immunofluorescence**
Cells were grown in microscope flasks (Nunc) to about 80% confluence. Each slide was given 2 Gy of either carbon ions or X-rays. After irradiation, the cells were fixed in 2% paraformaldehyde in PBS at an appropriate time point (these were as follows: 1, 2, 6, 12 and 24 h). For γH2AX and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) phospho T2609 immunostaining, the cells were permeabilised with 0.2% Triton-X, washed twice with ice-cold PBS and incubated with 1% Bovine Serum Albumin (BSA) in PBS for 1 hr, before being probed with anti-γH2AX antibody.
(Millipore, Watford, UK) and DNA-PKcs (phospho T2609) (Abcam, Cambridge, UK) both at a dilution of 1:200 in 1% BSA in PBS for 1 hr at room temperature. Cells were then washed three times with PBS before adding Alexa Fluor 488-conjugated goat anti-mouse Immunoglobulin G secondary antibody (Millipore) at a dilution of 1:400 in PBS for 1 hr, protected from light. Cells were washed three times with PBS before adding 0.5 μg/ml propidium iodide (PI; Invitrogen Life Sciences, Carlsbad, CA, USA) in PBS for 5 min. Finally, coverslips were mounted with 10 μl of ProLongGold anti-fade reagent (Invitrogen). Foci scoring was carried out using a in-house and a Nikon fluorescence microscope. At least 100 cells per sample were chosen at random on the slide and the γH2AX foci were counted by eye on single plane images.

Measuring nuclei area and peak fluorescence brightness

Cells were treated with 0.5, 5 or 10 μM of SAHA for 24 hr in 35 mm Petri dishes. The cells were then incubated for 1 hr using 2 μM Hoechst 33258 (Invitrogen) to stain the nucleus of live cells. A fluorescent microscope with a XY stage was used to take images of at least 1000 cell nuclei for each of the conditions. Cells were imaged using a EMCCD camera model C9100-13 (Hamamatsu, Tokyo, Japan), with an exposure time of 30 ms and gain of 3.0, with the fluorescent output of the mercury arc lamp set to 50%. Images were standardised by imaging an Yttrium Aluminum Garnet doped with Cerium (YAG:Ce) scintillator which fluoresces in both blue and green wavelengths. The intensity recorded from the scintillator was used to normalise fluorescent intensity across the field of view of the microscope, and to normalise between images. Pixels within cells above an 18% intensity threshold were recorded. The time duration of imaging all dishes was small, so degradation of the mercury arc lamp, or bleaching of the YAG:Ce scintillator over this time period can be assumed to be negligible. The images were ultimately analysed using a custom made software to measure the area and the peak intensity of the nuclei (Barber et al. 2007).

Statistical analysis

All of the experiments were repeated in triplicate on at least two separate days. The results are averaged and the standard error of the mean is given. All curve fittings were performed in OriginPro (Northampton, MA, USA) with an optimisation of the fit weighted to the variance of the data points.

Where appropriate, statistical significance was determined using a two-sample t-test and p-values less than 0.05 were considered significant.
The toxicity of SAHA to the cell lines (see Figure 1) was fitted with a Hill equation:

\[ y = V_{\text{max}} \frac{c^n}{k^n + c^n} \]

Where \( V_{\text{max}} \) is the saturation drug concentration, \( k \) is a constant which is half of the \( V_{\text{max}} \), \( c \) is the drug concentration, and \( n \) is the Hill coefficient describing cooperativity.

The Linear-Quadratic (LQ) equation was used to fit clonogenic survival data (see Figure 2):

\[ y = e^{-\alpha d - \beta d^2} \]

Where \( \alpha \) defines the linear component, \( \beta \) relates to the quadratic component, and \( d \) is the radiation dose.

A two component exponential model was used to fit the \( \gamma \)H2AX repair kinetics data (see Figure 3), adapted from Horn et al. (Horn et al. 2011). To calculate the half life of the fast (\( T_{1/2}\)Fast) and slow (\( T_{1/2}\)Slow) component, each component was multiplied by the natural logarithm of 2:

\[ y = y_0 + A_1 e^{-x/\tau_1} + A_2 e^{-x/\tau_2} \]

Where \( A \) is the amplitude of the curve, \( \tau_1 \) and \( \tau_2 \) are the mean lifetime constants and \( x \) is the time in hours.

The Sensitiser Enhancement Ratio (SER) was given using the following formula (see Table I):

\[ SER_{x\%} = \frac{\text{dose}_{x\%}(\text{no drug})}{\text{dose}_{x\%}(\text{drug})} \]

Where \( \text{dose}_{x\%}(\text{no drug}) \) is the radiation dose (Gy) required to produce \( x\% \) cell survival without drug and \( \text{dose}_{x\%}(\text{drug}) \) in presence of drug (i.e. SAHA). SER was calculated at doses related to surviving fractions of 37 and 50%.

The relative biological effectiveness at 10% survival (RBE\( _{10\%} \)) is given using the following formula:

\[ RBE_{10\%} = \frac{\text{dose}_{10\%} (X - \text{rays})}{\text{dose}_{10\%} (\text{Carbon ions})} \]

Where the dose is measured in Gy.

Results

SAHA sensitivity
Figure 1 shows the sensitivity of both cell lines, LN18 and U251, to continuous SAHA exposure with different concentrations. The Hill model fit to the data shows that LN18 cells are just over three times more sensitive than U251 with a half-maximal cytotoxic concentration (CC_{50}) of 9.77 ± 0.87 µM compared to 36.99 ± 0.88 µM for U251. However, treatment for 24 hr with 0.5 µM SAHA, rather than continuously, does not affect the plating efficiency for either cell line (data not shown). This non-toxic regime was used for all radiosensitisation experiments. Importantly, the concentration of 0.5 µM SAHA is clinically relevant, in accordance with the peak plasma concentration and the area under the plasma drug concentration-time curve measured after a daily dose of 500 mg (Kelly et al. 2005; Lee et al. 2012).

**X-ray and carbon ion sensitivity**

Figure 2 shows survival curves for both cell lines treated either with or without SAHA and irradiated with either X-rays or carbon ions. Table I shows the fits of these data to the LQ equation giving the α and β parameters.

Both cell lines have largely similar radiosensitivities to X-rays; however, they do also display some differences; U251 has a smaller α (and so a larger “shoulder”) and a slightly larger β parameter. In general, this means that U251 are more capable of fixing sub-lethal damage (attributed to the “shoulder”) but have a reduced ability to fix complex DNA breaks, attributed to a large β-parameter (Hall and Giaccia, 2006).

U251 is slightly more resistant to carbon ions than LN18. This is reflected by a lower RBE_{10%} of 3 ± 0.6 for U251 and 3.5 ± 0.6 for LN18 (see Table I). The survival curve for U251 again has a noticeable “shoulder”, whereas the LN18 data can be fitted using only the α parameter (i.e. no “shoulder”) which accounts for the slight difference in the RBE value.

**SAHA radiosensitising effect**

Table I shows the Sensitiser Enhancement Ratio (SER) for SAHA in combination with X-rays or carbon ions at 50% (SER_{50}) and 37% (SER_{37}) survival. For both cell lines and radiation qualities, radiosensitisation is greater at higher doses. Radiosensitisation with X-rays is very similar between cell lines with a SER_{50} of about 1.3. For LN18, X-rays produce more radiosensitisation than carbon ions (SER_{50} values being 1.31 and 1.19 for X-rays and carbon ions, respectively) whereas for U251
the effect is reversed (SER\textsubscript{50} values being 1.37 and 1.55 for X-rays and carbon ions, respectively). Similar effects are also suggested by the prolonged γH2AX foci expression.

**γH2AX foci repair kinetics**

The foci repair kinetics (Figure 3) after 2 Gy X-rays are similar between the two cell lines with both having a fast and slow foci repair half-life of between 0.5 and 1 hr and between 15 and 30 hr, respectively. The absolute numbers of foci with untreated cells are broadly similar too, starting with about 20 foci at 1 hr with no SAHA and finishing with fewer than 5 foci at 24 hr. However, both cell lines treated with SAHA consistently have on average about 2-5 more foci than their untreated counterparts. This is probably because cells treated with SAHA have a slightly larger $T_{1/2}\text{Slow}$ component, resulting in a slower foci repair rate.

The situation is different for cells irradiated with 2 Gy carbon ions. For LN18 cells, SAHA makes little difference to the repair kinetics and also does not increase the number of foci significantly. However for U251, SAHA increases the slow repair half-life from about 15 to 50 hr. This is reflected in the absolute number of foci remaining at 24 hr, where the SAHA-treated U251 cells have more than double the number of foci remaining compared to the untreated counterpart ($p=0.001$). Thus, overall, SAHA appears to slow the repair of DSB, indicated by the comparative longevity of foci. It should also be noticed that single exposure to 0.5 µM SAHA did not increase the background level of γH2AX foci up to 24 hr after treatment, which varies between 0.08 and 0.1 foci/cell for both cell lines.

The recruitment of DNA repair proteins at sites of DSB involves histone acetylation, ubiquitination and chromatin remodelling. To investigate how SAHA might affect this process, in particular foci formation, we assessed the number of both γH2AX and DNA-PKcs (phospho T2609) showing that both these proteins are similarly recruited to DSB in cells incubated with or without SAHA prior to irradiation with 2 Gy X-rays ($p>0.05$; Figure 3F).

*Effect of SAHA on Hoechst dye binding to DNA*

Measuring the nucleus area and the DNA peak fluorescence (maximum brightness) of nuclei indicates the extent of chromatin reorganisation and decondensation caused by SAHA. Generally, cells will stain heterogeneously with Hoechst dye, as denser chromatin (heterochromatin) regions will contain more dye.
Figure 4 shows boxplots of the variation in fluorescent peak intensity in the nucleus after treatment with various concentrations of SAHA, with each box representing about 1000 cells. If the peak intensity is high, there are regions in the nucleus which are highly compacted and concentrate the Hoechst dye. Overall, Figure 4 shows that the DNA dye intensity decreases with increasing concentrations of SAHA. This indicates that SAHA is relaxing the chromatin and creating a more homogeneous and decondensed chromosomal structure. In particular, LN18 cells have less bright regions than U251 cells, and also an incremental decline in the intensity with increasing concentration of SAHA. The situation is similar with U251 cells, although there is no significant difference between cells treated with 5 and 10 µM. The pertinent point is that 0.5 µM SAHA, which is used in the present paper in combination with radiation, significantly relaxes the chromatin (p << 0.05).

Discussion
The results presented here show that SAHA is an effective radiosensitiser with both X-rays and carbon ions. The greatest sensitivity is observed in U251 cells when irradiated with carbon ions. SAHA in combination with X-rays increases cell killing by about 30% for both cell lines compared to X-rays alone. While for carbon ions in combination with SAHA the cell killing is increased by cell about 20% for LN18 and 55% with U251 when compared to carbon ions alone.

The radiosensitising effect is in some measure related to unrepaired DNA DSB. γH2AX foci assays show that SAHA treated cells have consistently higher numbers of foci than their untreated counterparts. The main reason for this is probably the slower rate of DNA DSB repair when SAHA is present. The slower rate of repair is most evident for U251 cells treated with carbon ions and SAHA. Here, more than double the foci remain after 24 hr with the slow component of the repair rate more than three times slower compared to cells not treated with SAHA. This shows that U251 cells, particularly in the presence of SAHA, have difficulty in repairing the predominantly complex DNA strand breaks that result from carbon ion treatment. Nakajima et al. (2014) analysed the morphology, intensity and size of foci from 70 keV µm⁻¹ Carbon ions and 200 keV µm⁻¹ Fe ions. It was found that clusters of γH2AX foci radiated outwards from the original track caused by secondary short range delta-electrons. These foci are repaired with DNA non-homologous end-joining (NHEJ) kinetics in G1 phase, with the magnitude of complexity diminishing with time. This reflects the two component repair kinetics that we have shown in Figure 3.
The effect of carbon ions and SAHA in terms of γH2AX foci is not so pronounced on LN18 cells, which could be due to this cell line being more efficient at DSB repair in the presence of SAHA. However, the RBE\textsubscript{10} is slightly higher with LN18 cells compared to U251 (3.5 compared to 3.0) with a comparable difference in cells treated with SAHA. Intuitively, we should have expected U251 to be more sensitive to carbon ions owing to their slower DNA repair rate in presence of SAHA. However, 2 Gy of carbon ions (see Figure 2) killed the majority (> 95%) of the irradiated cells of both cell lines in either treatment scenario and therefore it was difficult to detect any variation induced by SAHA in terms of RBE\textsubscript{10} at very low survival levels.

Fluorescent dye incorporation into the nucleus with increasing concentrations of SAHA showed decreasing levels of DNA peak brightness, indicating substantial decondensation of the chromosomes and a more homogeneous chromatin arrangement. The chromatin structure and its arrangement in the nucleus directly influences the expression of genes (Felsenfeld and Groudine, 2003). The repeating unit of chromatin consists of approximately 146 base pairs of two superhelical turns of DNA wrapped around an octamer core of pairs of histones, H2A, H2B, H3 and H4. The histones can be modified including the acetylation of lysine, methylation of lysines and arginines and phosphorylation of serines. Histone deacetylases (HDAC), in part, determine the pattern of histone acetylation within the nucleus. Inhibition of HDAC, by agents such as SAHA, causes an accumulation of acetylated histones. Acetylated histones are associated with a neutralisation of the lysine positive charge leading to the chromatin becoming less dense. In general, this allows for greater access to promoter regions of genes and consequent upregulation in gene expression.

Growing evidence shows that HDACi selectively kill cancer cells at levels which are non-toxic to normal cells. This is because chromatin relaxation allows transcription of genes which induces growth arrest or apoptosis. Gui et al. (2004) showed that p21\textsuperscript{WAF1} (which regulates cell cycle checkpoints and ultimately proliferation) is upregulated in ARP-1 tumour cells in the presence of SAHA. This work was corroborated by Belluci et al. (2013) who found that activation of p21\textsuperscript{WAF1} requires acetylation of the histone H2A.Z. Moreover, Conti et al. (2010) found that SAHA can slow down replication forks, induce DNA damage and activate dormant origins of DNA replication, specifically decreasing the expression of HDAC3. Toth et al. (2004) showed that the HDACi trichostatin A (TSA) caused decondensation of interphase chromatin and increased apoptosis with HeLa cells. Interestingly, they showed that a number of HDAC must be inhibited simultaneously
and cooperatively to explain the apoptosis increase that was measured with increasing concentrations of TSA. Hence, it is highly likely that the toxicity seen with HDACi stems from genome wide action on the regulation of a number of genes simultaneously. About 20% of all known genes are affected by HDAC inhibition and the ratio of upregulation and downregulation is close to 1:1 (Minucci and Pelicci 2006). Following exposure to SAHA, gene expression analysis on colon cells and mesenchymal stem cells has revealed a wide range of upregulated and downregulated genes (Di Bernardo et al. 2010; LaBonte et al. 2009). While in general DNA repair proteins like Ataxia telangiectasia mutated (ATM), and Rad51 are downregulated inhibiting DNA repair efficiency, on the contrary cell cycle genes, such as p21, are generally upregulated by SAHA causing an aberrant cell cycle arrest that may lead to apoptosis (Richon et al. 2000).

Not only are promoters of genes more accessible in the presence of HDACi, but HDAC inhibition can also modify acetylation sites on non-histone proteins, changing their function. For example, Roy et al. (2005) showed that p53 can be stabilised in the presence of an HDACi (CG-1521) after acetylation, leading to increased cell cycle arrest and apoptosis in prostate cancer cells. Similarly, it has been found that SAHA blocks the protein Rad51 from co-localising with γH2AX DSB foci and thereby preventing repair (Chen et al. 2012). It was found that the Rad51 protein’s function itself was disrupted independently of the transcribed amount of protein available. Moreover, the baseline level of Rad51 was reduced with SAHA treatment. It was also found that SAHA selectively inhibited the homology-directed repair pathway, but interestingly, not the NHEJ pathway. Similarly, Harikrishnan et al. (2008) found that the HDACi, valproic acid, induced histone independent disruption which contributed to the radiosensitising effects on cells, although exactly which proteins were responsible for this effect were not identified.

Similarly to our results, Camphausen et al. (2004) used a HDACi, MS-275, and found that it radiosensitised U251 cells by about 30% when combined with X-rays. Moreover, a significantly greater number of cells were γH2AX positive at 24 hr after 5 Gy, although at 6 hr there were similar numbers of foci to the untreated counterparts. Interestingly, the radiosensitisation was greater for a prostate cancer cell line than the U251 cell line, although no reason was given for this. It was unclear whether MS-275 affected the acetylation status of H2AX, or whether an upstream component was disrupted so that DSB repair was impeded. In the light of Chen et al. work, it is likely that the HR pathway is disrupted, with Rad51 being impeded. Either way, it is clear
that cell lines are prone to DSB repair disruption by HDACi, and the extent of this is cell line dependant.

To our knowledge, there have only been two previous reports of HDACi and radiosensitisation with carbon ions. Kano et al. (2009) used cyclic hydroxamic-acid-containing peptide 31 (CHAP31) and 50 keV μm\(^{-1}\) carbons ions and showed about a 50% enhancement with squamous cancer cells. Importantly, they also showed that \(p21^{WAF1}\) was upregulated in the presence of CHAP31. Similarly, Oertel et al. (2011) combined SAHA and carbon ions (the LET was not given) and found similar levels of radiosensitisation between carbon ions and conventional X-rays. Moreover, they also found that \(p21^{WAF1}\) was upregulated in the presence of SAHA while p53 was unaffected. Cell cycle analysis revealed that cells were blocked in G1 or G2/M, while γH2AX foci repair kinetics showed cells with significantly more foci after SAHA and carbon treatment than their un-irradiated counterparts.

Substantial evidence supports the hypothesis that HDACi can sensitise cancer cells to ionising radiation, to greater or lesser extents depending on the cell line. The main reason for this appears to be the inability of treated cells to repair DNA breaks, probably by the interference with Rad51 and the HR pathway. Coupled with this is the upregulation of \(p21^{WAF1}\) in cancer cells with HDACi, causing cell cycle delay and increased propensity for apoptosis. The mechanism of radiosensitisation is probably related to a combination of both of these factors, and probably influenced by other disrupted pathways yet to be identified. In general, it does not appear that significant additional radiosensitisation is generally achieved using high-LET radiation over conventional X-rays. However, there is some evidence that complex DNA DSB caused by high-LET radiation or high doses of X-rays, are less easily fixed in the presence of an HDACi. Hence, the SER\(_{57}\) is less than that at SER\(_{50}\). In this way, the sensitising enhancement ratio at a clinically relevant dose (i.e. 2 Gy fraction) may have a higher efficacy with carbon ions than with X-rays, as high-LET radiation clusters DNA damage causing complex breaks.

**Conclusions**

In the present paper, it has been shown that SAHA is an effective radiosensitiser when used in combination with either X-rays or carbon ions on GBM cancer cells, and can enhance cell death up to 50% more than controls. Compared to X-rays alone, the combination of carbon ions and SAHA can increase the RBE\(_{10\%}\) by more than 3.5 times. This is a significant finding as GBM is one of the
most aggressive forms of cancer with a mean survival rate of about one year, even when treated with X-rays and TMZ. Thus, a combination of SAHA and radiation of either high or low LET quality might represent a potentially effective treatment strategy for patients with GBM, with the additional benefit that high LET radiation could limit normal tissue toxicity as normal tissues are receiving less radiation and therefore any possible SAHA-mediated radiosensitizing effect on normal cells is further reduced. The mechanism of radiosensitisation with SAHA is not completely clear, but the results presented in this paper show that it is partially due to the interference of DSB repair as demonstrated by greater γH2AX foci in SAHA-treated cells. However, the global effect that SAHA has on the nucleus, as exemplified by the decondensation of the chromatin, means that many possible pathways are affected which could lead to cancer cell radiosensitisation.

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Declaration of interest
The authors do not have any declaration of interest to report.
Figure legends

Figure 1: The sensitivity of two GBM cell lines (LN18 and U251) after continuous incubation with SAHA. The data have been fitted with the Hill equation. Error bars indicate the standard error of at least three independent experiments.

Figure 2: (A) LN18 and (B) U251 cells irradiated with either 250 kVp X-rays or 85 keV μm⁻¹ carbon ions with and without 0.5 µM SAHA. Error bars indicate the standard error of at least three independent experiments.

Figure 3: (A-D) DNA repair kinetics of cells irradiated with X-rays or carbon ions measured using γH2AX foci. The data are fitted with a two component exponential model (see methods). The approximated half-life of the repair time for the fast and slow component is given in the legend (A) LN18 cells irradiated with either 250 kVp X-rays or (B) 85 keV μm⁻¹ carbon ions with and without SAHA. (C) U251 cells irradiated with either 250 kVp X-rays or (D) 85 keV μm⁻¹ carbon ions with and without SAHA. Error bars indicate the standard error of at least three independent experiments. (E) Representative images of γH2AX formation in U251 cells at 0.5 hr after irradiation with 2 Gy X-rays with and without SAHA. (F) Representative images of DNA-PKcs (phospho T2609) foci in U251 cells at 0.5 hr after irradiation with 2 Gy X-rays with and without SAHA. Nuclei were stained with PI (red). The white scale bar is 10 µm.

Figure 4: The effect of SAHA on the binding of Hoechst DNA dye in the nuclei of (A) LN18 and (B) U251 cells with varying concentrations of SAHA. The fluorescent peak intensity measures the brightest collection of pixels in the nucleus, and is an indication of densely compacted chromatin. The boxes show the median, 25 and 75 percentiles, the whiskers and crosses show the 5 and 95, 1 and 99 percentiles respectively, and the squares show the mean. With both cell lines, the mean of the control and cells treated with 0.5 µM SAHA are significantly different at p = 0.05, using a Student’s t-test. (C) Representative images of Hoechst 33258-stained U251 cell nuclei treated with different concentration of SAHA (0, 5 and 10 µM). Nuclei within red squares were identified by the microscope system described in Methods. The white scale bar is 10 µm.
Table legends

**Table I:** Mean values and standard errors of $\alpha$, $\beta$, SER$_{50}$, SER$_{37}$ and RBE$_{10}$ estimated by fitting the cell survival data of Figure 2 to the LQ model.

Supplementary figure legends

**Supplementary Figure S1:** Representative images of DSB repair at 0.5, 2, 6, 12 and 24 hr post irradiation with 2 Gy Carbon ions with and without SAHA. Images were captured using a Nikon fluorescence microscope. The white scale bar is 10 µm.
References


Figures:

Figure 1

![Graph showing surviving fraction vs. dose of SAHA](image)
Figure 2

A. LN18

B. U251
Figure 3
Figure 4

A. LN18

B. U251

C. U251 untreated, 5 μM SAHA, 10 μM SAHA
### Supplementary Figure 1

<table>
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<th>Time</th>
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Table 1: Mean values and standard errors of α, β, SER\textsubscript{50}, SER\textsubscript{37} and RBE\textsubscript{10} estimated by fitting the cell survival data of Figure 2 to the LQ model.

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<th>LN18</th>
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<td>SER\textsubscript{37}</td>
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