Sensitization of glioblastoma cells to irradiation by modulating the glucose metabolism

Han Shen1*, Eric Hau1,2*, Swapna Joshi1, Pierre J Dilda3, and Kerrie L McDonald1

Authors and affiliations:

1Cure Brain Cancer Neuro-Oncology Group, Adult Cancer Program, Lowy Cancer Research Centre, Prince of Wales Clinical School, University of New South Wales, Sydney, 2052, Australia

2Cancer Care Centre, St George Hospital, Kogarah, NSW 2217, Australia.

3Tumour Metabolism Group, Adult Cancer Program, Lowy Cancer Research Centre, Prince of Wales Clinical School, University of New South Wales, Sydney, NSW 2052, Australia

*co-first author

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Corresponding author: Kerrie McDonald, Cure Brain Cancer Neuro-Oncology Group, Adult Cancer Program, Lowy Cancer Research Centre, Prince of Wales Clinical School, University of New South Wales, Sydney, 2052, Australia.

T: +61 2 9385 1471; F: +61 2 9385 1510; E-mail: k.mcdonald@unsw.edu.au

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**Abbreviations:** RT, radiotherapy; GBM, glioblastoma; PDK, pyruvate dehydrogenase kinase; DCA, dichloroacetate; ROS, reactive oxygen species; O$_2$, oxygen molecule; HIF, hypoxia inducible factor; OCR, oxygen consumption rate; ECAR, extracellular acidification rate.

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Abstract

Since radiotherapy (RT) significantly increases median survival in patients with glioblastoma (GBM), the modulation of radiation resistance is of significant interest. High glycolytic states of tumor cells are known to correlate strongly with radioresistance, thus the concept of metabolic targeting needs to be investigated in combination with RT.

Metabolically, the elevated glycolysis in GBM cells was observed post-RT together with upregulated hypoxia inducible factor (HIF)-1α and its target pyruvate dehydrogenase kinase 1 (PDK1). Dichloroacetate (DCA), a PDK inhibitor currently being used to treat lactic acidosis, can modify tumor metabolism by activating mitochondrial activity to force glycolytic tumor cells into oxidative phosphorylation. DCA alone demonstrated modest anti-tumor effects in both in vitro and in vivo models of GBM and has ability to reverse the RT-induced glycolytic shift when given in combination. In vitro, an enhanced inhibition of clonogenicity of a panel of GBM cells was observed when DCA was combined with RT. Further mechanistic investigation revealed that DCA sensitized GBM cells to RT by inducing the cell cycle arrest at the G2/M phase, reducing mitochondrial reserve capacity, and increasing the oxidative stress as well as DNA damage in GBM cells together with RT. In vivo, the combinatorial treatment of DCA and RT improved the survival of orthotopic GBM-bearing mice.

In conclusion, this study provides the proof of concept that DCA can effectively sensitize GBM cells to RT by modulating the metabolic state of tumor cells. These findings warrant further evaluation of the combination of DCA and RT in clinical trials.
Introduction

Glioblastoma (GBM) is the most malignant form of primary brain tumor in adults. Despite increasing attention on targeted therapeutics in the treatment of GBM, radiation therapy (RT) remains the most clinically effective treatment modality (1). However, RT only offers palliation, the efficacy of which is often limited by the occurrence of radioresistance, reflected as a diminished susceptibility of the irradiated cells to undergo cell death (2). To enhance the tumor cell sensitivity to RT, the mechanisms underlying radioresistance need to be further elucidated and strategies developed to overcome them.

When ionizing radiation passes the living tissue, the ionization of H₂O leads to the production of reactive oxygen species (ROS) that contain chemically active oxygen molecules leading to oxidative stress and DNA damage. Oxygen molecules (O₂) can stabilize the chemical composition of the DNA damage by reacting with the free radicals, such that O₂ chemically ‘fixes’ DNA damage. Unlike the balance achieved in normal tissues, the consumption of O₂ by tumor tissue is much higher than the O₂ supply from the surrounding blood vessels. Malignant solid tumors with inadequate blood supply and inconsistent perfusion therefore contain large portions of hypoxic cells which exhibit a high degree of resistance to chemoradiotherapy due in part to an increase of hypoxia inducible factor-1 (HIF-1) and expression of other cellular survival molecules (3). Radiation itself, has been shown to stabilize the activity of HIF-1, which in turn regulates a plethora of genes involved in angiogenesis, invasion, metabolism, and protection against oxidative stress (4). The residual tumor cells surviving after chemoradiotherapy eventually proliferate and lead to cancer relapse.

It has long been known that the metabolism of solid tumors is radically different from that in the corresponding normal tissues. Numerous studies have demonstrated that tumor cells
predominantly utilize glycolysis even in the presence of ample oxygen, referred as the Warburg effect. Using glycolysis provides a growth advantage for tumor cells and leads to malignant progression (5). GBM, like most malignant solid tumors, is highly glycolytic, producing large amounts of lactic acid as a metabolic by-product. It has been shown that tumors with high levels of glycolysis are less responsive to radiotherapy and behave more aggressively (6). More recent reports have identified the Warburg effect to be implicated in resistance to cytotoxic stress induced by either chemotherapy or RT (7). In this way, treatment methods which block or reduce glycolytic metabolism may increase tumor cell sensitivity to RT.

Under hypoxic conditions, HIF-1 causes an increase in its target gene pyruvate dehydrogenase kinase 1 (PDK1), which acts to limit the amount of pyruvate entering the citric acid cycle, leading to decreased mitochondrial oxygen consumption (8). These findings suggest that inhibition of PDK1 could alter the glucose metabolism and increase oxygen consumption of tumor cells, which would re-sensitize the tumor cells to RT. Dichloroacetate (DCA), a PDK inhibitor that has the potential for such metabolic targeting, has been shown to reverse the Warburg effect by shifting glucose metabolism from glycolysis to mitochondrial oxidation and to inhibit tumor cell growth (9, 10). By combining with RT, DCA has been demonstrated to enhance the radiosensitivity of several tumor types in vitro (11-13). Interestingly, a previous study using two human cancer cell lines (colon adenocarcinoma and GBM) demonstrated DCA sensitized the efficacy of RT in vitro but attenuated RT-induced tumor growth delay in vivo (colon adenocarcinoma model) (13). This paradoxical effect of DCA drove us to further investigate the efficacy and the mechanism of action of RT-DCA combination in both in vitro and in vivo GBM models. Therefore, in the present study, we first examined the hypothesis that RT promotes glycolytic metabolism, and then tested whether a reversal of the glycolytic phenotype will re-sensitize GBM cells to RT using DCA. The findings of this study may have important implications for clinical trials aimed at
preventing post-RT metabolic changes and increasing the therapeutic index of RT for GBM patients.

Materials and Methods

Cell Culture and Chemical

GBM cell lines (U87, U251, LN229, DBTRG) were purchased from ATCC. U87 and U251 were cultured in MEM (Gibco) with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. LN229 was cultured in DMEM (Gibco) with 10% FBS and 2 mM L-glutamine. DBTRG was cultured in RPMI1640 (Gibco) supplemented with 10% FBS, 2 mM L-glutamine, 25 mM HEPES and 1 mM sodium pyruvate. Cell lines were obtained within the past five years and authentication was not performed. A patient-derived GBM cell line RN1 (unmethylated, rs16906252 wild-type and p53 mutant) was kindly provided by our collaborative researchers at the Queensland Institute of Medical Research and was cultured in Advanced DMEM/F12 (Gibco) mixed with NeurobasalTM-A medium (Gibco) (1:1) supplemented with B-27 (1×), FGF (20 ng/mL) and EGF (20 ng/mL). Sodium dichloroacetate (DCA) was purchased from Sigma.

Cell cycle analysis

Cells were seeded in 6-well plates, followed by treatments as indicated. After treatments, cells were harvested and fixed in cold 70% v/v ethanol for at least 2 h. Fixed cells were washed with PBS and stained in the dark with a solution containing propidium iodide (10 µg/mL), Triton X-100 (0.1%) and RNAse (100 µg/mL) for 20 min at room temperature. DNA content was analyzed using a BD FACSCanto™ II flow cytometer and data analysis was performed using FlowJo (TreeStar Inc).
**Mitochondrial superoxide production assays**

Cells were seeded in 6-well plates, followed by treatments as indicated. MitoSOX Red (Invitrogen) was used to measure the level of mitochondrial superoxide production according to the manufacturer’s instructions. After staining, cells were trypsinized, centrifuged and resuspended in HBSS (1mL). Sytox Blue (Invitrogen; 1 μM) was added to counter-stain for non-viable cells. MitoSOX Red fluorescence was analyzed using BD FACSCanto™ II flow cytometer.

**Extracellular flux assay**

The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of GBM cells were determined using the XF Extracellular Flux Analyzer (Seahorse Bioscience). XF 24-well plates were coated with Matrigel (BD biosciences) to allow cells to attach for this assay. Briefly, each well of the 24-well plate was coated with 50 μL diluted Matrigel (1:50 in PBS) overnight. The next day, cells were plated at a density of 30,000 cells (U87) and 50,000 cells (RN1) per well and allowed to attach overnight in culture media. The following day the adherent cells were treated as indicated. On the assay day, cells were washed and fresh assay media was added. The cartridge was loaded to dispense three metabolic inhibitors sequentially as specific time points: oligomycin (inhibitor of ATP synthase, 1 μM), followed by FCCP (a protonophore and uncoupler of mitochondrial oxidative phosphorylation, 1 μM), followed by the combination of rotenone (mitochondrial complex I inhibitor, 1 μM) and antimycin (inhibitor of complex III, 1 μM). Specifically, the addition of oligomycin is used to measure the rate of proton leak across the inner mitochondrial membrane. The injection of FCCP is used to measure the rate of uncoupled respiration and is determined from the maximum respiration rate. The addition of rotenone/antimycin combination is to inhibit the transfer of electrons from iron-sulfur centers in complex I to ubiquinone and the oxidation of ubiquinol in the ETS, thereby inhibiting the oxidative phosphorylation. Basal OCR and ECAR were measured, as well as the changes in OCR caused by the addition of the metabolic
inhibitors described above. Several parameters were deducted from the changes in OCR and mitochondrial reserve capacity was calculated by subtracting basal respiration from maximal respiratory capacity as described previously (14). A mirror plate was set up and treated identically in parallel with the assay plate. At the end of the assay, cells from the mirror plate were harvested and the numbers of viable cells determined by flow cytometry were used to normalize the measurements.

**Western blotting**

Cells were seeded in 6-well plates, followed by treatments as indicated. Cells were lysed with RIPA buffer (Life Technologies), sonicated and centrifuged (14000 rpm, 10 min, 4°C). Protein concentration was measured using BCA Assay Kit (Pierce). Proteins (50 μg/sample) were separated via reducing 10% SDS-PAGE and standard western blotting procedures (15) were used to detect proteins of interest with the following primary antibodies: γ-H2AX (Cell Signaling Technology, CST #9718), HIF-1α (CST, #3716), PDK-1 (CST, #3820), β-actin (Abcam, ab8227). Images and densitometry were acquired by using ImageQuant TL software.

**Glucose metabolism PCR array**

Cells were plated in 6-well plates, followed by treatments as indicated. Total RNA from cells was extracted using RNeasy Mini Kit (Qiagen). 0.8 μg of total RNA was used for generating cDNA with RT² First Strand Kits (Qiagen), and was analyzed by a glucose metabolism PCR array according to the manufacturer's instructions using ViiA™ 7 Real-Time PCR System (Life Technologies). All data were normalized to expression of untreated control in the corresponding sample.
**Colony survival assay**

Cells were seeded into 6-well plates and allowed 24 h for attachment followed by treatments as indicated. Culture medium was changed after 24 h treatment. Plates were incubated for 2 weeks undisturbed. Colonies were gently washed with PBS followed by staining and fixation with crystal violet solution (0.5% in H₂O: Methanol 1:1) for 15 min. Stained colonies consisting of >50 cells were counted and the number was recorded. Plating efficiency was calculated as the number of colonies counted divided by number of cells seeded and normalized to the average plating efficiency of untreated samples. The average of these values was reported as ‘surviving fraction’. For the combination therapy of DCA and RT, the surviving fraction was normalized to DCA treatment alone.

**Mouse xenograft orthotopic model**

The procedures for developing an orthotopic brain tumor model were performed according to a protocol approved by the Animal Care and Ethics Committee of University of New South Wales. Briefly, female athymic nude mice (Balb/c, 8 weeks) were intracranially injected with 5 × 10⁴ U87 cells stereotactically in the right caudate putamen using the coordinates: 1 mm anterior, 1.5 mm lateral and 3.0 mm below from the bregma.

**DCA treatment and RT**

The U87-bearing mice were randomly divided into four groups of 8-10: vehicle treated control, radiation alone, DCA alone, and combination treatment. DCA was administered by oral gavage at 150 mg/kg/day from day 3 after tumor inoculation to the end of experiment. Mice in vehicle treated control and radiation alone arms received distilled water by oral gavage. Radiation was delivered to the whole brain of the mouse using a self-contained X-ray system (X-RAD 320) from day 13 after tumor implantation. During RT, mice were placed in a customized lead box shielding the body to allow radiation to be delivered directly to the
entire brain. The total radiation dose administered was 20 Gy at a clinically relevant 2 Gy/fraction schedule on 10 consecutive days. Two mice were randomly selected from each arm and culled on the day of RT completion. Their brains were harvested for H&E staining and immunohistochemistry examination. Mice were euthanized when they exhibited symptoms indicative of significant compromise to neurological function and/or a greater than 20% body weight loss. Animal survival was defined as the time euthanasia and survival curves were established by Kaplan-Meier estimator.

**Immunohistochemistry**

Tumor cell proliferation was estimated after H&E staining and by immunostaining for Ki-67. Excised tumors were fixed in formalin, embedded in paraffin and 5 µm sections were cut and mounted on Superfrost slides. Briefly, sections were deparaffinized in xylene, rehydrated in phosphate buffered saline using an ethanol gradient and a heat mediated antigen retrieval step was performed using the target retrieval solution (DAKO) at 95°C for 20 min. Sections were stained for proliferating tumor cells as follows: sections were incubated with primary Ki-67 antibody (DAKO) at 1:75 dilution for 1 h at room temperature and secondary antibody (DAKO) at 1:300 dilution for 1 h at room temperature. The proliferative index of tumor cells was determined by counting Ki-67 positive cells in three random 40x magnification fields per slice.

**Statistical analyses**

All analyses were performed using GraphPad Prism. Each independent experiment was performed with at least triplicate samples per treatment group. Results are expressed as mean ± s.d. of replicate values from three independent experiments or representative of three independent experiments presented as means ± s.d. of triplicate measurements. Statistical analysis was performed by two-way analysis of variance corrected by Dunnet’s
test or Student’s t test. Kaplan-Meier survival was compared using Log-rank (Mantel-Cox) test. All tests of statistical significance were two-sided and p values < 0.05 were considered statistically significant.

**Results**

*RT shifts the glucose metabolism from oxidative phosphorylation to glycolysis and the addition of DCA blocks RT-induced glycolytic phenotype in GBM cells*

We firstly examined the expression of a panel of key genes involved in the regulation and enzymatic pathways of glucose and glycogen metabolism. Compared to untreated control, we observed that most of the glycolytic genes were upregulated in U87 cells irradiated with 6 Gy at 4 h post-RT. In particular, all the isozymes of PDK (PDK 1-4) were upregulated from 1.26- to 3.38-fold (Figure 1 A, Table 1). To determine whether HIF-1α might be involved in this RT-induced glycolysis, western blotting was performed to examine the protein level of HIF-1α and its target PDK1. Compared to untreated control, treatment of U87 cells with 6 Gy RT significantly increased HIF-1α and PDK1 expression up to 2.47- and 1.42-fold respectively (Figure 1B). To further confirm the RT-induced glycolysis, two metabolic parameters, OCR and ECAR, were measured using an extracellular flux assay. The bioenergetic profiles were obtained 4 h post-RT with increasing RT doses. As shown in Figure 1C, the ECAR values of U87 cells after RT increased dose-dependently whereas a dose-dependent decrease in OCR was observed. By combining 10 mM DCA with RT, the RT-induced glycolytic rate of U87 cells was attenuated significantly (p<0.01) compared to cells treated with RT alone (Figure 1D). Similar results were also obtained for the primary GBM cell line RN1 (Figure 1E).
DCA treatment induces G2/M cell cycle arrest in GBM cells

We next further examined whether the proliferation arrest from DCA treatment was associated with induction of cell cycle arrest. Cells were treated with 25 and 50 mM DCA for 24 h, and cell cycle profiles were analyzed using flow cytometry. DCA treatment induced changes in the cell cycle profiles of all tested GBM cells (Figure 2A, B). Specifically, after 24 h of treatment with 25 mM DCA, there was a slight increase (not significant) in the cells in G2/M phase in U87 and U251 cells, and a 1.2-fold increase (p<0.001) in RN1 cells (Figure 2B). Significant increase in the mean percentage of all three tested cell lines in G2/M phase was observed when DCA dose was increased to 50 mM (Figure 2B). When compared with untreated control (U251 8.8%, U87 15.2%, RN1 14.1%), DCA treatment increased the proportion of cells at G2/M phase to 35.5%, 34.7% and 45.5%, respectively. A corresponding decrease in cells in G1 and S phases in all GBM cell lines was also observed (Figure 2B).

DCA treatment depletes mitochondrial reserve capacity in GBM cells

Because ionizing radiation exerts its cytotoxic effects predominantly through the generation of free radicals and subsequent oxidative stress, we investigated whether the mitochondrial reserve capacity of GBM cells would be altered by DCA treatment (Figure 3A). After 24 h treatment with 10 mM DCA, the basal OCR of DCA treated cells increased from ~4200 pmole/min/10^6 cells (untreated control) to ~5400 pmole/min/10^6 cells (Figure 3B). By sequential injecting oligomycin, FCCP, the mixture of antimycin and rotenone, complete mitochondrial profile was further revealed in U87 and RN1 cells (Figure 3B and C). As DCA treatment did not obviously affect the maximal respiratory capacity of tumor cells, the mitochondrial reserve capacity of DCA treated cells significantly decreased (p<0.05) (Figure 3D). By increasing DCA concentration to 20 mM, the reserve respiratory capacity was further significantly reduced to ~1960 pmole/min/10^6 cells and ~490 pmole/min/10^6 cells in U87 and RN1 cells respectively (p<0.001) (Figure 3D).
**RT in combination with DCA induces ROS production in GBM cell**

As DCA increased oxidative stress by directing pyruvate into mitochondria, increasing electron transport chain activity and thus generating more ROS, we proposed that this increased ROS could potentially further increase the ROS induced by RT. To test this hypothesis, GBM cells (U251, U87 and RN1) were treated with DCA, RT alone and the combination of two therapies followed by mitochondrial ROS level detection. For U251, 24 h DCA treatment (25 and 50 mM) led to a dose-dependent increase in mitochondrial ROS levels by 1.5~2-fold compared to untreated control (Figure 4A). On the other hand, the mitochondrial ROS level of U251 cells treated with RT increased 1.3- (12 Gy) and 1.5-fold (18 Gy) compared to that of control cells (Figure 4A). These values further increased when the DCA treatment was combined with RT. Specifically, the combination of DCA (25 and 50 mM) and RT (12 and 18 Gy) dose-dependently boosted the mitochondrial ROS level of U251 up to ~3.5-fold as opposed to control cells (Figure 4A). Compared to U251, U87 cells are more resistant to DCA treatment and RT. Significant elevation of the MitoSox Red fluorescence was only induced with high dose DCA treatment (50 mM) and RT (18 Gy) alone (p<0.01) (Figure 4B). A further increase in mitochondrial ROS production was observed when combined treatment was administered (Figure 4B). Similar results were obtained from RN1 cells (Figure 4C). These data suggest a higher capacity of the combination to induce mitochondrial ROS generation in comparison with individual treatments alone.

**DCA augments the DNA double-strand breaks induced by RT**

As we observed the combination of RT and DCA induced higher mitochondrial levels of ROS than each treatment alone, a further experiment was conducted to investigate the change in γ-H2AX levels, a hallmark of DNA damage, after treatment of RT and DCA. Western blotting for γ-H2AX production was measured 30 min after RT (4 Gy), 24 h DCA treatment (50 mM) and the combination (pre-treatment with DCA for 24 h followed by RT). For U251, both RT
and DCA treatment increased γ-H2AX production (4.75 and 2.71-fold) compared to untreated control, whilst the combination further induced γ-H2AX production up to 8.93-fold (Figure 4D). Compared to U251, U87 is more resistant to the RT and DCA treatment. Either RT or DCA treatment as monotherapy showed similar levels of DNA damage with 1.29- and 1.82-fold γ-H2AX production. When the two treatments were combined, significantly higher γ-H2AX production (2.41-fold) was observed (Figure 4D). Similar results were also obtained from RN1 with 2.08-fold, 1.52-fold and 3.48-fold increase for RT, DCA and the combination respectively (Figure 4D).

**DCA treatment synergizes with RT to impair the clonogenicity of GBM cells**

Clonogenic survival assays were conducted to examine whether DCA is a radiosensitizing agent for GBM cells. U251, LN229 and DBTRG were irradiated with 2, 4 and 6 Gy after 6 h of pre-treatment with DCA (U87 and RN1 were not selected for this assay as they were not able to form clear colonies to be counted). As a single agent, DCA treatment induced a dose-dependent clonogenic inhibition in these GBM cell lines (Table S1). The clonogenicity of these cells was further impeded when treated with combination compared to DCA and RT alone (Figure 5A, B and C). For U251 and DBTRG cells, DCA significantly synergized with all of the three tested RT doses to reduce the colony formation (Figure 5A, C), while for LN229 cells we observed an additive effect when DCA was combined with 2 and 4 Gy RT and synergy with 6 Gy (Figure 5B).

**The combination therapy extends the median survival of GBM orthotopic model**

We determined the efficacy of RT-DCA combination using a xenograft orthotopic mouse model bearing U87 cells. The dosage of RT (20 Gy in 10 fractions) and DCA (150 mg/kg/day) were selected based on our previous studies showing either of them significantly delayed the growth of orthotopic U87 xenograft (data not shown). No toxicity was noticed in any of the
treatment arms. Compared to vehicle treated control (median survival time, MS=29 days), DCA alone (MS=31 days) prolonged the median survival of orthotopic model by 2 days (p<0.01) whilst a more significant prolongation of median survival (p<0.001) was found in RT alone (MS=38 days) arm (Figure 5D). Moreover, an even longer survival benefit was observed in RT-DCA combination arm (MS=43 days) compared to each treatment alone (p<0.001 vs RT arm) (Figure 5D). The histological analysis of the vehicle and treated tumors at day 23 confirmed tumor formation (Figure 5E) and indicated significant reductions in the proliferation of the tumor cells, as reflected in number of cells staining for the proliferation marker Ki-67 (Figure 5F, G).

Discussion

In the current study, several distinct lines of evidence indicate the efficacy of RT was significantly enhanced by DCA treatment in GBM cells. Our findings first confirmed an initial finding from others that a shift of glucose metabolism from oxidative phosphorylation to glycolysis occurred in GBM cells following RT, despite ample oxygen availability, which strongly support a switch to aerobic glycolysis. By combining DCA with RT, this glycolytic phenotype was reversed. We demonstrated that DCA alone moderately induced proliferation arrest at the G2/M phase and reduced the mitochondrial reserve capacity, indicative of sensitizing GBM cells to the effect of RT. Additionally, DCA and RT worked synergistically to induce elevated mitochondrial ROS level as well as γ-H2AX production, further confirming the mechanism of action of this combination. Finally, the clonogenicity of GBM cells was impaired by the combination of DCA and RT and the survival of an orthotopic mouse model was extended significantly by the combination therapy compared with each single treatment arm. All these findings above provide the evidence that modulating the glucose metabolism may serve as an effective approach to sensitize these malignant cells to RT.
RT kills cancer cells primarily through free radical-induced DNA damage (16). However, it has been shown this cornerstone of treatment acts as a double-edged sword. RT has been linked with HIF-1 activation/stabilization, which in turn activates transcription of numerous genes involved in angiogenesis, proliferation, pH regulation, and glycolytic metabolism (17-19). In particular, HIF-1 not only initiates transcription of genes that encode transporters and enzymes regulating glucose metabolism, but also activates the PI3K/AKT pathway that is involved in the regulation of metabolic shift to aerobic glycolysis. The underlying mechanism by which RT activates HIF-1 was mostly investigated using in vivo preclinical model. It has been thought that HIF-1 expression increases after RT due to reoxygenation and ROS elevation that induce HIF-1α stabilization (20). We confirmed here using cultured GBM cells that HIF-1α was activated post-RT as well as a panel of glycolytic genes. Interestingly, the upregulation of PDK1, a crucial mitochondrial enzyme that plays an important role linking glycolysis to oxidative phosphorylation and a direct target of HIF-1α (21), was also observed.

To further confirm this finding, we measured the glucose metabolism using an Extracellular Flux Analyzer. A dose-dependent increase in the acid production was observed and a decline in the oxygen consumption occurred simultaneously.

It has been demonstrated that the glycolytic metabolism in malignancies highly correlates with radioresistance (22). Tumor cells predominantly using glycolysis counter the direct and indirect action of RT, that is RT-induced free radical and oxidative stress, by upregulating the endogenous antioxidant capacity through accumulation of pyruvate, lactate, and the redox couples glutathione/glutathione disulfide and NAD(P)H/NAD(P)+ (23). These macromolecules are also the products of glycolytic pathway which constitute an intracellular redox buffer network effectively scavenging free radicals and ROS, thus blunting the efficacy of RT. In this way, it is a potential strategy to overcome radioresistance by modulating tumor glucose metabolism and the cellular redox status. GBM is a deadly brain tumor for which there are limited therapies and chemoradioresistance remains a serious problem to be
conquered. Altered glucose metabolism in GBM has been extensively investigated *in vitro* (5, 24, 25) and, more recently, *in vivo* in patients (26) and in human orthotopic GBM models (27). These studies established that glucose is oxidized in the citric acid cycle in addition to confirming that there is a significant fraction of glucose that is shunted to lactate generation. Most recently, the same group of investigators published data in the orthotopic models and in patients demonstrating that acetate is also being oxidized in the citric acid cycle, which further confirms the significant role of mitochondria in glucose metabolism of GBM cells.

After exposing to ionizing radiation, the uncoupling of oxidative phosphorylation occurred (28), which in turn elevates the glycolytic rate, providing cancer cells with numerous advantages via the Warburg effect (29). In this respect, reversal of the Warburg effect offers dual therapeutic advantages by not only enhancing the effect of ionizing radiation through induction of oxidative stress but also lowering acid production resulted from the uncoupling of oxidative phosphorylation.

DCA is a small molecule PDK inhibitor that can penetrate blood-brain-barrier and reverse the Warburg effect in cancer cells (30). It has been demonstrated with moderate anti-tumor activity (31-33) and to induce apoptosis in tumors of GBM patients by restoring the mitochondrial activity (34). Several studies have also used it as a radiosensitizer in lung (11), colorectal (13) and prostate cancer cells (12) *in vitro*. In the present study, we employed DCA as a radiosensitizer in both *in vitro* and *in vivo* GBM models. DCA was selected for combination with RT, as we proposed that by reversing the glycolytic phenotype with DCA and directing more pyruvate into mitochondrial oxidation (to produce more ROS), tumor cells would be more sensitive to RT. We found that concurrent use of DCA with RT indeed prevented the increase in glycolytic rate observed following RT alone. Our previous study demonstrated that DCA effectively induced a dose-dependent proliferation arrest of *in vitro* cultures of GBM cells but had no significant effect on non-cancerous cells (15). This result is in line with most of the studies that investigated the efficacy of DCA demonstrating that a
therapeutic window exists although suprapharmacological level of DCA (5-50 mM) is necessary to halt the growth of cancer cells in vitro (9, 33, 35, 36). In the current study, we further analyzed the cell cycle distribution after DCA treatment showing that DCA induced G2/M arrest and decreased the cell proportion in S phase. These findings are consistent with some (35, 37), but not all studies (12, 38), indicating not all cancer types share the same mechanism of action after DCA treatment. As cancer cells are most sensitive to RT in the G2/M phase of the cell cycle whilst most resistance in the S phase (39-41), our results suggest that DCA treatment can sensitize GBM cells to RT by altering cell cycle distribution. Moreover, the mitochondrial reserve capacity, a measure of the ability of cells to resist oxidative stress (42), was reduced dose-dependently after DCA treatment, which further confirms the role of DCA as a potential radiosensitizer to GBM cells, even if the inhibition of glycolysis may also partly contribute to its role of radiosensitization in this setting.

As ROS are synthesized during mitochondrial respiration, an increased rate of mitochondrial oxidation might exacerbate the ROS-induced DNA damage caused by RT (43, 44). It has been reported that DCA treatment induced ROS production both in vitro and in vivo (45). Recent evidence also indicated that DCA increases autophagy in associate with increased ROS production in colorectal cancer cells (46). Given ROS plays an important role in their individual mode of action of DCA and RT, we thus combined DCA with RT and demonstrated that the combination treatment not only increased the ROS production but also caused higher level of DSBs than each treatment alone. These findings were further verified by our in vitro efficacy study demonstrating the clonogenicity of GBM cells was impaired synergistically when DCA was combined with RT. Ultimately, an orthotopic GBM mouse model was used to test this hypothesis in vivo. A survival advantage was observed in the combination treatment arm compared with either single treatment arm and the results were confirmed with histological examination showing a low percentage of Ki-67 positive cells in the combination treated tumors. These data again support our conclusion that DCA
treatment may overcome the radioresistance of GBM cells by reversing the glycolytic phenotype whilst synergizing with RT by stimulating the production of ROS. Notably, the discordance in the radiosensitizing effect of DCA was observed when it was tested in vitro and in vivo. In vitro, the effective radiosensitizing dose of DCA ranges from 25 to 50 mM evidenced from the result of colony surviving assay as well as our mechanistic studies. In contrast, the efficacy of radiotherapy was sensitized by DCA with 150 mg/kg/day in vivo that gives much lower serum level of DCA. This finding is consistent with previous reports describing this anti-metabolic compound has better in vivo efficacy than in vitro activity (37, 47, 48). The reasons for the limited antitumor effect of DCA with clinically relevant doses in vitro may lie in the complex cellular physiology and the immense excess of metabolites existing in cell culture media (47). Due to the fact that DCA has better in vivo activity than in vitro activity, it also has been suggested that there might be unique metabolic features and special growth pattern for solid tumors that are difficult to recapitulate in vitro and may be critical in determining the efficacy of this class of drugs (47).

DCA has been used as an orphan drug for various acquired and congenital disorders of mitochondrial metabolism for decades and has recently been proved to be feasible and well-tolerated in patients with recurrent malignant gliomas in a recent phase I clinical trial (49). In addition, a recent study has tested the efficacy of DCA in a small cohort of GBM patients, suggesting metabolic modulation through PDK inhibition as a novel therapeutic strategy for the treatment of this devastating brain tumor (34). This study also observed that apoptosis was further increased in the GBM stem cells treated with DCA plus temozolomide (TMZ), indicating DCA may potentiate the effect of standard chemotherapy. Moreover, the use of DCA reverses the post-RT glycolytic changes such that the efficacy of RT is enhanced without adding extra toxicity. Taken together, our findings warrant further evaluation of the combination of DCA and RT/TMZ in clinical trials for newly diagnosed GBM patients.
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References


Table 1 The fold-change of glucose/glycogen metabolism-related genes in irradiated U87 cells in comparison with untreated control. This table shows the corresponding results of the heat map (Figure 1A).

**Glucose metabolism**- Glycolysis: ALDOA, ALDOB, ALDOC, BPGM, ENO1, ENO2, ENO3, GALM, GCK, GPI, HK2, HK3, PFKL, PGAM2, PGK1, PGK2, PGM1, PGM2, PGM3, PKLR, TPI1. Gluconeogenesis: FBP1, FBP2, G6PC, G6PC3, PC, PCK1, PCK2; Regulation: PDK1, PDK2, PDK3, PDK4, PDP2, PDPR. TCA cycle: ACLY, ACO1, ACO2, CS, DLAT, DLD, DLST, FH, IDH1, IDH2, IDH3A, IDH3B, IDH3G, MDH1, MDH1B, MDH2, OGDH, PC, PCK1, PCK2, PDHA1, PDHB, SDHA, SDHB, SDHC, SDHD, SUCLA2, SUCLG1, SUCLG2; Pentose Phosphate Pathway: G6PD, H6PD, PGLS, PRPS1, PRPS1L1, PRPS2, RBKS, RPE, RPIA, TALDO1, TKT.

**Glycogen metabolism**- Synthesis: GBE1, GYS1, GYS2, UGP2; Degradation: AGL, PGM1, PGM2, PGM3, PYGL, PYGM; Regulation: GSK3A, GSK3B, PHKA1, PHKB, PHKG1, PHKG2.
**Figure 1** RT-induced glycolytic phenotype is blocked by the addition of DCA. (A) A panel of glycolytic genes in U87 cells was upregulated 4 hr after RT 6 Gy. (B) RT 6 Gy induced HIF-1 expression as well as its target PDK1. Western blots presented are representative of 3 independent experiments. (C) RT induced a decrease in OCR whereas an increase in ECAR dose-dependently. The addition of DCA significantly inhibited the RT-induced acidification rate in (D) U87 and (E) RN1 cells. Due to a high cell seeding density (30,000-50,000 cells per well) used in this assay, relatively high doses of RT (6-20 Gy) were used here to induce ECAR in a short timeframe. Results are representative of three independent experiments and presented as means ± s.d. of 3-5 measurements. *p<0.05, **p<0.01, ***p<0.001, #p<0.01 vs RT 20 Gy.

**Figure 2** DCA induces cell cycle arrest in GBM cells. (A) Representative flow cytometry histograms showing cell cycle distribution in untreated control and 24 hr DCA-treated RN1 cells. (B) Cell cycle analysis showing the percentage of cells in G1, S and G2/M phase in untreated- and DCA-treated GBM cells (U87, U251 and RN1). Results are presented as means ± s.d. of three experiments, performed in triplicates. *p<0.05, **p<0.01, ***p<0.001.
Figure 3 DCA depletes the mitochondrial reserve capacity of GBM cells. (A) A schematic diagram extracted from Seahorse Bioscience demonstrating the use of specific inhibitors to determine the mitochondrial profile is shown. After 3-5 measurements of base line OCR, oligomycin, FCCP, and the mixture of antimycin A and rotenone were injected sequentially with measurements of OCR recorded after each injection. ATP-linked OCR and the OCR due to proton leak can be calculated using the basal and the oligomycin-sensitive rate. Injection of FCCP is used to determine the maximal respiratory capacity. Injection of antimycin A and rotenone allows for the measurement of OCR independent of Complex IV. The spare respiratory capacity (mitochondrial reserve capacity) is calculated by subtracting the basal from the maximal respiration. Bioenergetic profiles of (B) U87 and (C) RN1 were measured using sequential injection of oligomycin (1 μM), FCCP (1 μM), and the mixture of antimycin A (1 μM) and rotenone (1 μM). (D) The mitochondrial reserve capacity of untreated control and DCA-treated GBM cells (U87 and RN1) is shown. Seahorse XF24 analyzer protocol included 3 min of mixing, 2 min of waiting, and 3 min of measurement for each measurement. Results are representative of three independent experiments and presented as means ± s.d. of 3-5 measurements. *p<0.05, ***p<0.001.

Figure 4 The combination of RT and DCA induces oxidative stress and DNA double-strain breaks. RT, DCA and combination induced a dose-dependent increase in the fluorescence of MitoSOX Red in (A) U251, (B) U87 and (C) RN1. (D) The expressions of γ-H2AX, a hallmark of DNA double-strain breaks, in GBM cells (U251, U87 and RN1) are shown after treated with RT, DCA and their combination. Western blots presented are representative of 3 independent experiments. Results are presented as means ± s.d. of three experiments. **p<0.01, ***p<0.001; #p<0.05, ##p<0.01 vs untreated control; $p<0.05, $$p<0.01 vs untreated control.
**Figure 5** Efficacy of RT in combination with DCA *in vitro* and *in vivo*. Clonogenic survival assay showing the combination of DCA and RT impairs the clonogenicity of (A) U251, (B) LN229 and (C) DBTRG cells. The surviving fraction curves of RT/DCA combination have been normalized to the curves of DCA-only. Any combination curve overlapping with the RT-only curve indicates additive effect, while any combination curve under the RT-only curve suggests synergistic effect. Results are representative of three experiments and presented as means ± s.d of triplicate measurements. (D) Kaplan-Meier survival analysis of xenograft orthotopic U87 model treated with DCA (150 mg/kg/day), RT (20 Gy in 10 fractions), and RT-DCA combination. (E) Representative images from H&E staining of mouse brain containing U87 tumor. t, tumor; b, normal brain parenchyma. (F) Representative images (40×) from Ki-67 staining of U87 orthotopic tumor. (G) Quantification of Ki-67 positive cells in U87 orthotopic tumors treated with 150 mg/kg/day DCA, 20 Gy RT, and the combination treatment. Results are expressed as the means ± s.d., with n = 6 in each group. ns, not significant, **p<0.01, ***p<0.001. #p<0.001 vs RT 20 Gy.
Figure 1

A Visualization of log2(Fold Change)

B Control RT 6Gy

<table>
<thead>
<tr>
<th>MW</th>
<th>HIF-1α</th>
<th>PDK-1</th>
<th>elF4E</th>
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<tr>
<td>140</td>
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<td>47</td>
<td>1.00</td>
<td>1.42</td>
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</tr>
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C U87

D U87

E RN1

OCR (pmol/min/10⁶ cells)

ECAR (pmol/min/10⁶ cells)
Figure 2

A

Untreated control

DCA treated

B

U87

U251

RN1

Cell cycle distributions (% of cells)

Control

DCA 25 mM

DCA 50 mM

Cell cycle distributions (% of cells)

Control

DCA 25 mM

DCA 50 mM

Cell cycle distributions (% of cells)

Control

DCA 25 mM

DCA 50 mM

**

***
Figure 3

A

Mitochondrial Respiration

Oligomycin  
FCCP  
Antimycin A  
& Rotenone  

Spare Respiratory Capacity  
Maximal Respiration  
ATP Production  
Proton Leak  
Basal Respiration  
Non-mitochondrial Respiration

OCR [pMoles/min]

0  10  20  30  40  50  60  70  80  90  100  110

TIME [minutes]

B

U87

OCR [(pmol/min/10^6 cells)]

0  20  40  60  80  100  120  140  160

Time (min)

C

RN1

OCR [(pmol/min/10^6 cells)]

0  1000  2000  3000  4000  5000

Time (min)

D

Mitochondrial reserve capacity

OCR [(pmol/min/10^6 cells)]

0  1000  2000  3000  4000

U87  RN1

Control  DCA 10mM  DCA 20mM

Control  DCA 10mM  DCA 20mM

*  ***  *
Figure 4

A) U251

B) U87

C) RN1

D) U251, U87, RN1
Figure 5

A. U251

B. LN229

C. DBTRG

D. Survival Percentage

E. Histological Images

F. Immunohistochemical Images

G. KI67 Positive Cells
Sensitization of glioblastoma cells to irradiation by modulating the glucose metabolism

Han Shen, Eric Hau, Swapna Joshi, et al.

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