Original Contribution

Dichloroacetate alters Warburg metabolism, inhibits cell growth, and increases the X-ray sensitivity of human A549 and H1299 NSCL lung cancer cells

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A B S T R A C T

We investigated whether altering Warburg metabolism (aerobic glycolysis) by treatment with the metabolic agent dichloroacetate (DCA) could increase the X-ray-induced cell killing of the radiation-resistant human non-small-cell lung cancer (NSCLC) cell lines A549 and H1299. Treatment with 50 mM DCA decreased lactate production and glucose consumption in both A549 and H1299, clear indications of attenuated aerobic glycolysis. In addition, we found that DCA treatment also slowed cell growth, increased population-doubling time, and altered cell cycle distribution. Furthermore, we report that treatment with 50 mM DCA significantly increased single and fractionated X-ray-induced cell killing of A549 and H1299 cells. Assay of DNA double-strand break repair by neutral comet assays demonstrated that DCA inhibited both the fast and the slow kinetics of X-ray-induced DSB repair in both A549 and H1299 NSCL cancer cells. Taken together the data suggest a correlation between an attenuated aerobic glycolysis and enhanced cytotoxicity and radiation-induced cell killing in radiation-resistant NSCLC cells.

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1. Introduction

Lung cancer is the most common cancer in the world and is the second largest cause of cancer death in the USA [1,2]. It comprises non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). NSCLC is responsible for 85% of lung cancer. Because the early onset of NSCLC is asymptomatic, it is often diagnosed in its late clinical stage when surgical resection is not an option. Consequently, this subset of patients has a very poor prognosis and their 5-year overall survival (OS) outcome is 10%.

For these advanced stage patients, and early stage NSCLC patients who are medically inoperable because of cardiopulmonary comorbidities, radiation treatment is possible. Unfortunately, conventional external beam radiotherapy of 1.8–2.0 Gy per fraction for a total dose range of 45–66 Gy has only improved 5-year OS rate to 20% [3–5]. However, the use of stereotactic body radiation therapy (SBRT) has shown promise as a clinical therapy for extracranial tumors including NSCL lung cancer [3,4,6–9]. SBRT involves giving large 15–20 Gy radiation doses per fraction delivered over 3–5 sessions. The high dose radiation has an ablative effect on the targeted tumor and its steep dose gradient limits damage inflicted on surrounding normal tissues [4]. Despite these advancements in technology, SBRT is not feasible if the NSCLC tumor is large and or centrally located within 2 cm of the bronchial tree due to morbidity and the twofold increase in severe grade 3 toxicities over peripheral tumors [3,4,9,10].

An alternative methodology for these NSCLC patients with large tumors is to combine targeted and untargeted chemotherapy drugs with radiation to increase the tumor-killing efficacy with a lower radiation dose, and thereby perhaps limit damage to normal
tissue surrounding their NSCLC tumors [11–13]. However, a number of studies strongly suggest that targeting abnormal cancer cell metabolism such as aerobic glycolysis or Warburg metabolism with metabolic agents should be investigated as a potential therapeutic approach in combination with radiation or chemotherapy [14–21]. It has long been known that most cancer cells utilize aerobic glycolysis or Warburg metabolism instead of oxidative phosphorylation for cell energy production [22–25]. Aerobic glycolysis not only increases glucose consumption and lactate production but has also recently been shown to facilitate cell proliferation via biomass incorporation of nucleotides, amino acids, and lipids [25]. Dichloroacetate (DCA) is a synthetic small molecule used to treat hereditary metabolic or cardiovascular diseases [26,27]. It has high bioavailability with 20% of systemic DCA bound to plasma protein [26,27]. DCA has been shown to be able to partially revert aerobic glycolysis in cancer cells and thereby lower glucose utilization and decrease lactate production [18,19,21,28–30]. Data indicate that DCA reduces lactate production in cancer cells by inhibiting the mitochondrial enzyme pyruvate dehydrogenase kinase that results in an (unphosphorylated) active pyruvate dehydrogenase complex (PDC) that increases pyruvate oxidation in the mitochondria, promotes glucose oxidation, and decreases lactate production in the cell cytoplasm, resulting in an overall increase in oxidative phosphorylation [19,26,30]. However, the true potential for metabolic drugs such as DCA may be as an adjunct to chemotherapy and radiation in multimodality cancer treatment, although to date results have been mixed [14–21,31].

In this report, we investigated whether the metabolic drug DCA could suppress human NSC lung cancer growth and increase the X-ray-induced cell killing of the radiation-resistant NSCLC lines A549 and H1299. A549 is p53 WT, Kras (G12S) mutated, while H1299 is p53 null and Kras WT. We examined the effects of treatment with DCA on cell growth, colony formation, cell cycle stage, X-ray sensitivity, and repair of DNA double-strand breaks (DSB) in both A549 and H1299 NSCLC cells. We report here that DCA sensitizes these NSCLC cells to X-ray-induced killing through cell cycle redistribution and inhibition of DNA double-strand break repair.

2. Materials and methods

2.1. Cell culture

Cells in exponential growth phase were harvested and plated in T-75 and T-25 flasks (Corning) for stock propagation and experiments, respectively, by our standard methods [11,32,33]. The flasks were placed in a 37 °C incubator with 5% CO2 levels and 85% humidity (Forma Steri-Cult HEPA Class 100 CO2 incubator, Thermo Scientific). The NSCLC cell lines A549 (p53 wild type, KRAS mutated) and H1299 (p53 null, KRAS wild type) were obtained from Dr. John Turchi, Indiana University School of Medicine. They were cultured in DMEM media (1X; Mediatech) supplemented with 10% FBS (Hyclone) and 1% penicillin/streptomycin (Mediatech). The ATCC immortalized bronchial epithelial cells (NL-20 ATCC CRL-2503) were propagated in Ham’s F12 media supplemented with 1.5 g/L sodium bicarbonate, 2.7 g/L glucose, 2.0 mM L-glutamine, 0.1 mM nonessential amino acids, 0.005 mg/ml insulin, 10 ng/ml epidermal growth factor, 0.001 mg/ml transferrin, 500 ng/ml hydrocortisone, and 4% FBS. All experiments below were performed in triplicate.

2.2. Glucose and lactate assays

DCA has been shown to be able to partially revert aerobic glycolysis in some cancer cells which results in lower glucose utilization and decreased lactic acid production [28,29]. To test whether DCA treatment of A549 and H1299 cells would alter glucose utilization or lactic acid production, the A549 or H1299 cells were plated at 2 × 105 cells per flask and returned to the incubator. After 24 h to allow for cell attachment and growth, various amounts of stock DCA solution were added to the A549 and H1299 flasks to achieve 0 or 50 mM DCA concentration and the flasks were incubated at 37 °C for 48 h. At 48 h time point, aliquots of the growth media were then removed from duplicates of each treatment group of A549 and H1299 cells, and glucose and lactate levels were measured by standard colorimetric assay kits for glucose (BioVision, K606-100) and for lactate (BioVision, K607-100). The flasks of cells were trypsinized and counted, and total number of cells was used to normalize absolute glucose and lactate values.

2.3. Plating efficiency/colony formation assays and population doubling time

Cells were seeded in T-25 flasks for the survival curve assays. A 0 or 50 mM DCA was introduced 24 h later. Cells were harvested at 24 and 48 h of DCA incubation along with parallel control 0 mM DCA and counted using a Beckman’s 2 coulter counter. A growth curve of cell counts versus time was generated for treated and nontreated samples at 24, 48, 72, and 96 h. The growth curves for A549 and H1299 cells with 0 and 50 mM DCA were graphically analyzed and the population doubling times determined by our standard methods [11,32,33]. To assess the effect of DCA on plating efficiency (PE), i.e., colony formation, adherent cells from the A549 and H1299 treatment flasks above were trypsinized, counted, and plated at appropriate numbers for colony formation assays by our standard methods [11,32,33]. The colonies were allowed to grow for 10 days before fixation with 70% ethanol followed by crystal violet staining (3%). Stained colonies with at least 50 cells per colony were scored. PE was defined as the ratio of scored colonies to number of cells plated [11,32,33].

2.4. Apoptosis (annexin V) and necrosis (SYTOX AADVanced)

Cells were plated and DCA-treated as in the propidium iodide (PI) staining protocol above. After DCA incubation, cells were trypsinized, counted and resuspended in annexin V buffer at 1×106/ml for AV and SYTOX staining (Invitrogen A35156). The volume of annexin V-PB and SYTOX AADVanced stock solutions used were determined using staurosporine as a stress inducer. They were respectively 4 and 2 μl for H1299 and 5 and 2 μl for A549. Annexin V incubation was for 15 min (room temperature, RT) for both cell lines. Cells were then washed and resuspended (1×106 cells/ml) in annexin V buffer. SYTOX was added for 2 min (RT) and assayed immediately with flow cytometry (LSRII, BD Biosciences).

2.5. Flow cytometry-based cell cycle distribution analysis with propidium iodide with an anti-phospho-histone H3 antibody

A549 and H1299 were plated at 1 × 106 cells per T-75 flask and allowed to grow overnight. Cell seeding number for ATCC2503 was 3 × 105 in T-75 flasks. A 50 mM DCA or control vehicle (water) was then introduced for 17 h. They were then trypsinized, counted, and fixed in 1:1 ratio of 200 proof ethanol and chilled PBS, and then placed in –20 °C freezer for 1 week. After pelleting at 1000 rpm (15 min) and PBS wash, cells were resuspended at 2 × 106/ml of PBS/0.1% Triton X-100, RNase A-treated (2 mg), and incubated with 0.40 ml of 500 μg/ml propidium iodide (Fluka 81845) for 30 min at room temperature. Cell samples were then placed on ice. Cell cycle flow cytometry analysis was performed on a LSRII flow cytometer.
immediate plating, 2.5 Gy. The experimental groups were as follows: Control, H1299 cells after fractionated or split-dose X-ray exposure, A549 cells after fractionated or split-dose X-ray exposure, A549 cells after 2.5 Gy with immediate plating and split-dose repair experiments.

2.6. Western blots

Cells were seeded at $1 \times 10^6$ in T-25 flasks and after 17 h of incubation with 0 or 50 mM DCA, they were washed in PBS and then lysed in RIPA buffer (0.5% NP-40, 0.5% SDS, 0.15 M NaCl, and 0.05 M Tris, pH 8) containing protease and phosphatase inhibitor cocktail (Pierce 78441). The lysate was scraped, collected into Eppendorf tubes, and sonicated on ice. Following centrifugation at 10,000 rpm (10 min), the supernatant was collected and used for protein estimation (Pierce 23236, Coomassie Plus Bradford assay) and fractionation. The immunoblot was probed with antibodies against cyclin E, cyclin B, cyclin D, cdk1 (phospho-Tyr15), cdk4 and 6 (Cell Signaling), anti-phospho-histone H3 (clone 3H10, Millipore), internal loading control was beta-tubulin. The specified protein bands were indirectly detected using fluorescent dye-labeled Alexa Fluor 750 goat anti-rabbit IgG (Invitrogen, A-21039) or goat anti-mouse IRDye (Odyssey, 926-32210) with the Odyssey Classic IR Imager (LI-COR Biosciences).

2.7. Clonogenic survival assays and X-ray survival curve construction

A549 or H1299 cells were plated into T-25 flasks, and unless indicated otherwise, the DCA dose used for the X-ray experiments was 0 or 50 mM DCA. Fresh media containing 0 or 50 mM DCA was introduced to the plated flasks daily for the next two days. On the second day postplating, cells were irradiated with 0, 2, 3, or 5 Gy with 160-kVp X-rays produced by a 160-kVp Faxitron X-ray machine (Faxitron Biopics, LLC). The X-ray machine had a 0.5 mm of Cu filtration and the source to target distance was 33 cm, which resulted in a dose rate of 62.8 Gy/min. The specimens were allowed to recover for 24 h and the adherent cells were trypsinized, counted, and plated at appropriate numbers for colony formation assays by our standard methods [11,32,33]. The colonies were allowed to grow for 10 days before fixation with 70% ethanol followed by crystal violet staining (3%). Stained colonies with at least 50 cells per colony were scored. PE, defined as the ratio of scored colonies to number of cells plated, was normalized to the respective control PE to give the surviving fraction (SF). All SFs (except for the initial DCA IC$_{50}$ determination) were corrected for DCA toxicity. This normalization measures the enhanced cell killing from radiation alone rather than the additive killing from combined drug and radiation [11,32,33].

2.8. Immediate plating and split-dose repair experiments

To investigate the effect of DCA on cell killing in A549 and H1299 cells after fractionated or split-dose X-ray exposure, A549 or H1299 cells were plated into T-25 flasks and incubated with 0 or 50 mM DCA. The experimental groups were as follows: Control, 50 mM DCA only, 5 Gy with immediate plating, 5 Gy with DCA and immediate plating, 2.5 Gy x 2 split dose with or without DCA and a 4 h incubation at 37 °C between the 2.5 Gy radiation doses to allow for repair. All cells were irradiated on ice to prevent repair during irradiation [11,32,33].

2.9. Neutral comet assays

To obtain a direct quantitative assay on DNA double-strand break (DSB) repair, neutral comet assays were performed with a commercial kit by our established methods [Trevigen 4250-050-k] [11,32,33]. At 24 h after plating, cells were treated with 0 or 50 mM DCA for 24 h, followed by irradiation at 20 Gy on ice. Postirradiation, the chilled cell culture supernatant was replaced with 37 °C 5% CO$_2$ preequilibrated media and incubated at 37 °C. Cells were harvested at time points from 0 to 3 h. After a chilled PBS wash, cells were embedded in agarose, lysed, and subjected to neutral electrophoresis. The gel was stained with SYBR green and imaged with a Nikon N-E fluorescence microscope under 10× magnification. The olive tail moments of the comets were analyzed using the TriTekCometScore Freeware program (www.autocomet.com). Average olive tail moment values [36] were obtained from at least 110 cells per sample.

2.10. Statistical analysis

Statistical significance was analyzed using the unpaired Student's t test for 3–6 independent replicates per assay (Prism 6.0e, GraphPad Software, Inc.). Error bars denote standard deviation. P values of 0.05 or less were considered statistically significant.

3. Results

3.1. DCA alters lactate production and glucose consumption in A549 and H1299 cells

Treatment of A549 and H1299 lung cancer cells with 50 mM DCA suppressed the production of lactate and reduced the consumption of glucose relative to controls in a statistically significant manner (Student's t test, P < 0.05) in both lung cancer cells except for glucose consumption in A549 which only approached statistical significance (P = 0.065) (Fig. 1). These results are consistent with published literature on DCA treatment shifting metabolism in select cancer cell types from aerobic glycolysis (Warburg metabolism) to oxidative phosphorylation [14-19,28-30]. In addition, preliminary studies of both A549 and H1299 with other concentrations of DCA indicated that 50 mM DCA was the approximate IC$_{50}$ value for both A549 and H1299 cells (Supplemental Fig. 1S). Based on these results, we performed all remaining studies with A549 and H1299 lung adenocarcinoma cells with 50 mM DCA. A very recent study by Shavit et al. in 2015 has confirmed similar IC$_{50}$ values in vitro for both A549 and H1299 cells [31].

3.2. DCA slows cell growth and increases the population doubling time of A549 and H1299

Growth curves of A549 and H1299 cells were performed with 0 and 50 mM DCA (Fig. 2, bottom left and right panels). We found that treatment of A549 and H1299 cells with DCA significantly increased population doubling times by ~twofold from 21 ± 2 to 46 ± 4 h for A549 and from 18 ± 3 to 38 ± 3 h for H1299 (Fig. 2, top left and right panels, *P < 0.05). Since our previous studies have shown that increases in cell population doubling times may be partially due to a reduction in clonogenic capacity, i.e., lower plating efficiency [11,32,33], clonogenic assays with and without DCA were performed.

3.3. DCA reduces plating efficiency/clonogenic capacity but does not induce apoptosis or necrosis in A549 or H1299 cells

To definitely determine the extent to which treatment with
50 mM DCA altered A549 or H1299 plating efficiency and/or mode of cell death, we plated A549 and H1299 cells into groups of T25 flasks and after 24 h postplating added 50 mM DCA to the flasks for two additional days. Forty-eight hours of 50 mM treatment with DCA significantly reduced plating efficiency of A549 and H1299 cells from 0.63 ± 0.06 (63%) and 0.70 ± 0.09 (70%) to 0.30 ± 0.04 (30%) and 0.43 ± 0.03 (42%), respectively (Fig. 3, top panels, *P < 0.05). However, flow cytometry-based apoptosis assay by annexin V and necrosis assay by measured by SYTOX AAD-advanced DNA staining indicated that the increase in cell death was not due to induction of either apoptosis or necrosis (Fig. 3, bottom panels).

3.4. DCA induces altered cell cycle distributions in A549 and H1299 cells

To investigate whether the increased cell population doubling times in both A549 and H1299 also resulted in altered cell cycle distributions, flow cytometry analysis with PI staining was performed and the percentages of cells in G1, S, and G2/M phase are shown in Fig. 4 (top panels). DCA treatment reduced the percentage of the S phase in both A549 and H1299 cells from 20% to 5% and 7%, respectively (*P < 0.05). In A549, treatment with 50 mM DCA induced a significant increase in the percentage of cells in G1 phase from 54% to 80% (*P < 0.05). In H1299, treatment with 50 mM DCA induced a significant increase in G2/M phase from 22% to 54% (*P < 0.05). Additional data, confirming the alterations in cell cycle by DCA in both A549 and H1299 cells, can be seen in Fig. 5 (top panels). Taken together, the data suggest that DCA may have induced altered cell cycle distributions or cell cycle arrest at the G1/S and G2/M checkpoints, perhaps through alterations of cyclins and the associated cycle-dependent kinases which were investigated below.

Fig. 4 bottom left panels show representative Western blots for G1 and S phase cyclins D and E as well as their associated cyclin-dependent kinase Cdk4 for A549 control and A549 50 mM DCA-treated cells. Cdk6, another G1/S cyclin-dependent kinase, was not detectable on Westerns in either A549 or H1299 cells and therefore not shown. The cell cycle analysis above suggested that treatment of A549 cells with DCA induced a large increase in the percentage of cells in G1 phase, perhaps due to an accumulation of cells at the G1/S checkpoint. Western blots and densitometry from three independent experiments for the G1/S cyclins (cyclin D and cyclin E) as well as their associated cyclin-dependent kinase 4 (Cdk4), which are required for exit from G1 and entry into and through S phase [37–45], were investigated to test this hypothesis (Fig. 4, bottom left panels). The data show that treatment with DCA reduced cyclin D, cyclin E, and cdk4 to 65%, 40%, and 69%, respectively, versus A549 control cell levels (t test, *P < 0.05). Taken together, these data suggest that A549 cells treated with DCA were accumulating at the G1/S border and not entering S phase (Figs. 4 and 5, additional data also in Supplemental Fig. 2S). By contrast, cell cycle analysis of H1299 cells treated with DCA indicated that DCA induced a significant increase in the percentage of G2/M cells perhaps due to accumulation at the G2/M checkpoint. Western blots and densitometry from three independent experiments for the G2-associated cyclin B and its associated G2 cyclin-dependent kinase 1 (Cdk1), which are both required for progression through G2 and entry into mitosis [37–45], were performed to test this hypothesis (Fig. 4, bottom right panels). The data showed that treatment of H1299 cells with DCA reduced both cyclin B and cdk-1 (specifically the phosphorylated tyrosine form

50 mM DCA induced a significant increase in G2/M phase from 22% to 54% (*P < 0.05). Additional data, confirming the alterations in cell cycle by DCA in both A549 and H1299 cells, can be seen in Fig. 5 (top panels). Taken together, the data suggest that DCA may have induced altered cell cycle distributions or cell cycle arrest at the G1/S and G2/M checkpoints, perhaps through alterations of cyclins and the associated cycle-dependent kinases which were investigated below.

Fig. 1. DCA alters lactate production and glucose consumption in A549 and H1299 cells. A549 and H1299 NSCLC cell cultures were treated with 0 or 50 mM DCA for 48 h and media glucose and lactic acid were then measured as ng/100 cells (*P < 0.05). Additional data, confirming the alterations in cell cycle by DCA in both A549 and H1299 cells, can be seen in Fig. 5 (top panels).
of cdk1) to 25%* and 26%**, respectively, versus untreated control H1299 cells (t test, *P < 0.05). These data therefore support our proposal that H1299 cells treated with DCA are accumulating at the G2/M border and not entering M phase. To further test this hypothesis, we performed additional PI-based flow cytometry assays with the addition of an antibody against phospho-histone H3, a mitosis-specific marker [34,35,46,47], in A549 and H1299 cells treated with 0 or 50 mM DCA. The data clearly show that the majority of the increase of the percentage of cells in G2/M phase is due to an increase in G2 cells and not cells in mitosis especially in the DCA-treated H1299 cells (Fig. 5, top and bottom panels, additional data in Supplemental Fig. 2S). By contrast, treatment of ATCC 2503 immortalized bronchial epithelial cells with an IC50 dose of DCA did not significantly reduce the percentage of radiation-resistant S phase cells (bottom panel, Supplemental Fig. 4S).

3.5. DCA increases the X-ray sensitivity of A549 and H1299 NSCLC cells

We initially performed DCA and X-ray dose escalation studies by irradiating A549 NSCLC cells with 0, 3, or 5 Gy of 160 kVp X-rays with and without treatment with 17, 37, and 50 mM DCA (Supplemental Fig. 3S). Any toxicity from DCA was normalized out in the X-ray survival curves to directly test whether treatment with DCA sensitized the NSCLC cells to X-ray-induced cell killing. These preliminary studies showed that DCA increased the X-ray sensitivity of A549 cells in a DCA and X-ray dose-dependent manner. The degree of sensitization was most pronounced at 5 Gy with 50 mM DCA. We therefore performed all additional X-rays studies with 50 mM DCA and clearly showed that a 50 mM DCA dose reduced the size of the X-ray survival curve shoulder and increased the steepness of the survival curve of A549 and H1299 cells (Fig. 6). Survival curve analysis indicates that treatment of H1299 cells with 50 mM DCA increased their X-ray sensitivity above 2 Gy by increasing the steepness and slightly reducing the shoulder of the H1299 survival curve (Fig. 6, right panel). Survival curve analysis indicates that treatment of H1299

Fig. 2. DCA slows cell growth and increases the population doubling time of A549 and H1299. A549 and H1299 cells were plated into groups of T-25 flasks and allowed to grow at 37 °C in a standard CO2 cell incubator. Twenty-four hours after initial plating 0 or 50 mM DCA was added to groups of flasks for both cell lines. Three flasks from each treatment group of A549 and H1299 cells were removed at 24 h intervals and the total cell counts were determined on days 1–4 postplating. Growth curves were plotted (bottom two panels) and the cell population doubling times (n = 6 experiments, mean ± SD) were graphically determined (top two panels). *t tests indicated that differences in cell population doubling times for A549 and H1299 treated with 0 or 50 mM DCA were significant (P < 0.05).
cells with 50 mM DCA reduced the \(D_0\) of H1299 cells from 2.6 to 1.7 Gy resulting in a DMF of 1.5 (2.6/1.7 Gy = 1.5). The DMF at the 10% survival level for the H1299 cells treated with DCA was 1.5 (7.5/5.5 Gy = 1.4). Treatment of an immortalized normal bronchial cell line ATCC 2503 with 50 mM DCA showed no evidence of increased X-ray-induced cell killing by DCA (Supplemental Fig. 4S, top panels).

3.6. DCA inhibits split-dose repair and enhances fractionated dose X-ray killing

To investigate if DCA increased the radiation sensitivity of A549 and H1299 lung cancer cells by inhibiting repair or split-dose recovery, we compared survival of A549 and H1299 cells given as a single dose of radiation (5 Gy) with or without DCA to those cells treated with two 2.5 Gy fractions of X-rays that were given 4 h apart with or without DCA (Fig. 7, top panels). Again, treatment of A549 and H1299 cells with DCA significantly reduced survival after a single 5 Gy X-ray dose and immediate plating (* indicating \(P < 0.05\)). Fractionating the X-rays into two 2.5 Gy doses to allow recovery/repair between the two fractions in A549 and H1299 cells significantly increased the surviving fraction compared to the single 5 Gy treatment (Fig. 7, top panels, ** and *** indicating \(P < 0.05\)). However, treatment of A549 and H1299 with DCA completely inhibited the (2.5 Gy + 4 h + 2.5 Gy) split-dose repair/recovery, reducing the surviving fraction comparable to that of the 5 Gy with DCA (Fig. 7, top panels, * indicating \(P < 0.05\)).

3.7. DCA inhibits DSB repair in A549 and H1299 NSCLC cells

The split-dose fractionation experiments presented above have been shown to be reasonable surrogate measurements of DNA single- and double-strand break repair [11,32,48–50]. However, the neutral comet assay has been shown to be a more direct method for investigating the induction and repair of DNA DSB in mammalian cells [11,36,51]. We performed neutral comet assays with A549 and H1299 cells irradiated with 20 Gy of X-rays and incubated with 0 or 50 mM DCA from 0.5 to 3 h postirradiation (Fig. 7, bottom panels). Comparison of the mean olive tail moments, an indication of the amount of DNA DSBs remaining versus time postirradiation, show that incubation with DCA slowed X-ray-induced DNA DSB repair down and resulted in consistently larger mean olive tail moments in DCA treated cells compared to 20 Gy irradiated controls (Fig. 7, bottom panels, * indicating \(P < 0.05\)). The 0 Gy DCA treated controls in both A549 and H1299 exhibited very similar olive moment to 0 Gy controls (data not shown). Taken together, the data suggest that treatment with DCA inhibits X-ray-induced DNA DSB repair in A549 and H1299 cells.
and shown above each protein band. Each protein band was measured. The mean B, and pTyr DCA ([26,27]). DCA has been shown to be able to partially revert aerobic metabolism agents that has high bioavailability and is currently being used to treat hereditary metabolic or cardiovascular diseases. The cell cycle experiments ([26,27]) indicate that targeting cancer cell Warburg metabolism with metabolic agents that has therapeutic potential especially in combination with radiation or chemotherapy [14–19,31]. DCA is a synthetic small molecule that has high bioavailability and is currently being used to treat hereditary metabolic or cardiovascular diseases [26,27]. DCA has been shown to be able to partially revert aerobic glycolysis in cancer cells in which the compound both lowers glucose utilization and decreases lactic acid production [18,19,28–30]. Studies indicate that DCA reduces lactate production by inhibiting the mitochondrial enzyme PDC kinase that results in an (unphosphorylated) active PDC complex that can partially restore mitochondrial glucose oxidative phosphorylation by DCA [28,29,31].

We then showed that treatment of A549 and H1299 NSCLC lung cancer cells and also appeared to at least partially reduce glucose consumption (Fig. 1). This is consistent with published data that some cancers that exhibit the glycolytic phenotype [22–25] can be partially reverted to mitochondrial glucose oxidative phosphorylation by DCA [28,29,31].

We then showed that treatment of 50 mM DCA to the A549 and H1299 NSCLC cells with DCA significantly increased cell population doubling time (Fig. 2). The data suggest that the increased population doubling time of A549 and H1299 cells induced by DCA can partially be explained by decreased colony formation or clonogenic capacity that appears not to be due to an increase in either apoptosis or necrosis (Fig. 3) and therefore most likely due to an increase in mitotic cell death/catastrophe in the daughter cell progeny of the DCA-treated cells. However, DNA flow cytometry analysis of cell cycle phases also suggests that DCA appeared to increase population doubling times by slowing cell cycle progression and reduction of the percentage of cells in S phase in both A549 and H1299 cells through induction of a G1/S block in A549 and a G2/M block in H1299 cells (Figs. 4 and 5, top panels). Western blot studies of cell cycle control proteins suggest that treatment of A549 cells with DCA reduces the amounts of cyclin D and cyclin E and the associated cyclin-dependent kinase Cdk4 and supports the induction of a G1/S block by DCA that prevents A549 cells from entering S phase (Fig. 4, and Supplemental data in Fig. 25). By contrast, Western blot studies of G2 and M cell cycle proteins in H1299 cells indicate that treatment of H1299 cells with DCA reduces the amount of cyclin B, which would support the induction of a possible G2/M block (Fig. 4, middle and bottom panels). Interestingly, analysis of the cyclin B-associated cyclin-dependent kinase, Cdk1 (specifically the phosphorylated tyrosine inactive form of Cdk1), clearly showed that pTyr-Cdk1 is reduced in H1299 cells treated with DCA, suggesting that the cells are not blocked at the G2/M border but perhaps are being blocked in mitosis. However, additional DNA flow cytometry studies with the mitosis-specific marker, the phosphorylated form of histone H3, clearly show that the

**4. Discussion**

Data suggest that cancer cells use aerobic glycolysis or Warburg metabolism to facilitate cell proliferation via biomass incorporation of nucleotides, amino acids, and lipids [22–25]. Some studies indicate that targeting cancer cell Warburg metabolism with metabolic agents has therapeutic potential especially in combination with radiation or chemotherapy [14–19,31]. DCA is a synthetic small molecule that has high bioavailability and is currently being used to treat hereditary metabolic or cardiovascular diseases [26,27]. DCA has been shown to be able to partially revert aerobic glycolysis in cancer cells in which the compound both lowers glucose utilization and decreases lactic acid production [18,19,28–30]. Studies indicate that DCA reduces lactate production by inhibiting the mitochondrial enzyme PDC kinase that results in an (unphosphorylated) active PDC complex that can partially restore mitochondrial glucose oxidative phosphorylation by DCA [28,29,31].

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We then showed that treatment of A549 and H1299 NSCLC cells with DCA significantly increased cell population doubling time (Fig. 2). The data suggest that the increased population doubling time of A549 and H1299 cells induced by DCA can partially be explained by decreased colony formation or clonogenic capacity that appears not to be due to an increase in either apoptosis or necrosis (Fig. 3) and therefore most likely due to an increase in mitotic cell death/catastrophe in the daughter cell progeny of the DCA-treated cells. However, DNA flow cytometry analysis of cell cycle phases also suggests that DCA appeared to increase population doubling times by slowing cell cycle progression and reduction of the percentage of cells in S phase in both A549 and H1299 cells through induction of a G1/S block in A549 and a G2/M block in H1299 cells (Figs. 4 and 5, top panels). Western blot studies of G1 and S cell cycle control proteins suggest that treatment of A549 cells with DCA reduces the amounts of cyclin D and cyclin E and the associated cyclin-dependent kinase Cdk4 and supports the induction of a G1/S block by DCA that prevents A549 cells from entering S phase (Fig. 4, and Supplemental data in Fig. 25). By contrast, Western blot studies of G2 and M cell cycle proteins in H1299 cells indicate that treatment of H1299 cells with DCA reduces the amount of cyclin B, which would support the induction of a possible G2/M block (Fig. 4, middle and bottom panels). Interestingly, analysis of the cyclin B-associated cyclin-dependent kinase, Cdk1 (specifically the phosphorylated tyrosine inactive form of Cdk1), clearly showed that pTyr-Cdk1 is reduced in H1299 cells treated with DCA, suggesting that the cells are not blocked at the G2/M border but perhaps are being blocked in mitosis. However, additional DNA flow cytometry studies with the mitosis-specific marker, the phosphorylated form of histone H3, clearly show that the
DCA-treated H1299 cells are not entering mitosis and are indeed being blocked at the G2/M border, probably as a result of the observed decrease in cyclin B (Fig. 5).

The above changes in both cell growth and cell cycle distribution in A549 and H1299 cells treated with DCA suggested that DCA may alter the X-ray sensitivity of these two NSCL cancer cells by a reduction in radiation-resistant S phase cells which we and others have shown to be associated with increased radiation sensitivity [11,32,33]. We found that treatment with IC50 doses of DCA significantly and synergistically increased X-ray-induced cell killing in both A549 and H1299 cells (Fig. 6), but did not increase the X-ray-induced cell killing of normal human bronchial epithelial cells ATCC 2503 (Supplemental Fig. 4S). A very recent study by Shavit et al. in 2015 has shown that a 1 h pretreatment of A549 and H1299 cells with similar IC50 doses of DCA also increases X-ray-induced cell killing [31]. It is unclear whether similar mechanisms of radioresitization by DCA that we have observed with a 48 h treatment of DCA are involved since 1 h pretreatment with DCA most likely would not alter the cell cycle distribution of either A549 or H1299.

Our data suggest that the increased X-ray sensitivity induced by a 48 h treatment with 50 mM DCA can be partially explained by alterations of cell cycle in A549 and H1299 cells. However, reductions in the shoulder of an X-ray survival curve have been strongly correlated by our laboratory and other investigators with reduced split-dose repair capacity [11,32,48–50]. Indeed, we clearly show here that treatment of A549 and H1299 cells with DCA reduced both split-dose repair capacity (Fig. 7, top panels) and DNA double-strand repair (Fig. 7, bottom panels). DNA double-strand break repair measured by comet assay indicated that DCA attenuated both the fast and the slow kinetics of DSB repair in both cell lines compared to irradiation alone (Fig. 7, bottom panels).

Our observation that treatment with DCA can enhance X-ray-induced cell killing in human cancer cells is in agreement with other investigators who have shown evidence of enhancement of X-ray-induced cell killing by DCA treatment in human colon adenocarcinoma, glioblastoma, and prostate cancer cells [14,17]. In addition, as stated above, a very recent study has now shown that a 1 h pretreatment with similar IC50 doses of DCA also increases X-ray-induced cell killing of A549 and H1299 cells [31]. Since all of the above studies involve either different DCA concentrations and/or different drug incubations times it is difficult to determine whether mechanisms similar to those we have observed, i.e., altered cell cycle distribution and inhibition of DNA repair, are involved in the reported enhancement of X-ray-induced cell killing by DCA by other laboratories. However, our data strongly suggest that alteration of DNA double-strand break repair by DCA may explain our observed enhancement of X-ray-induced cell killing in A549 and H1299 cells and future studies will investigate how treatment with DCA alters DNA double-strand break repair in A549 and H1299 NSCLC cells. It is possible that a reduction in cellular glucose uptake by DCA may result in a reduced nucleotide pool, which may account for the slowing of DNA DSB kinetics.

**Fig. 5.** Induction of altered cell cycle redistributions in A549 and H1299 cells confirmed by additional flow cytometry analysis of phospho-histone H3, a mitosis-specific marker. A549 and H1299 cells were plated into groups of T-25 flasks and after 24 h were treated by adding 0 or 50 mM DCA to the flasks. After 17 h of 0 or 50 mM treatment with DCA single cell suspensions were prepared for DNA flow cytometry analysis after propidium iodide (PI) staining with or without the addition of the antibody against the phospho-histone H3 mitotic marker. The percentage of cells in G1, S, G2/M or G2 and M phases for A549 and H1299 cells were measured and the average values are shown (n = 3 experiments, mean ± SD) (PI only, top panels, PI with anti-phospho-histone H3 antibody, bottom panels). *t tests indicated significant differences in the percentage of G1, S, G2/M, or G2 and M cells in A549 and H1299 treated with 0 and 50 mM DCA (P < 0.05).
Fig. 6. DCA increases the X-ray sensitivity of A549 and H1299 NSCLC cells. A549 and H1299 cells were plated into groups of T-25 flasks and after 24 h were treated by adding 0 or 50 mM DCA to the flasks. After 24 h of 0 or 50 mM treatment with DCA, groups of flask of each cell type and treatment group were irradiated with 0, 2, 3, or 5 Gy of 160 kVp X-rays and then returned to the incubator for 24 h of growth. From each cell type and experimental group single cell suspensions were prepared and plated for standard colony formation assay to determine the surviving fraction. Surviving fractions at 0, 2, 3, and 5 Gy for A549 cells treated with 0 and 50 mM DCA (left panel) and H1299 cells (right panel) are shown (*n=6 experiments, SF mean ± SD). It is important to note that any toxicity from DCA was normalized out in the X-ray survival curves to directly test whether treatment with DCA sensitized the NSCLC cells to X-ray-induced cell killing. *t tests indicated that differences in surviving fraction at these X-rays doses for A549 and H1299 treated with 0 or 50 mM DCA were significant (*P<0.05).

Fig. 7. DCA inhibits split-dose repair and enhances fractionated dose X-ray killing in A549 and H1299 NSCLC cells (top panels). A549 and H1299 cells were plated into groups of T-25 flasks and after 24 h 0 or 50 mM DCA was added to the flasks. After 24 h of 0 or 50 mM treatment with DCA, groups of flask of each cell type and treatment group were irradiated with either a single dose of X-rays (5 Gy) or two 2.5 Gy fractions of X-rays that were given 4 h apart. The surviving fractions after 5 Gy, 5 Gy + DCA, 2.5 Gy × 2, and 2.5 Gy X2 + DCA for A549 cells (top panel) and H1299 (bottom panel) are shown (*n=3–6 experiments, mean ± SD). *t, **, ***t tests indicated that differences between 5 Gy versus 5 Gy + DCA, 5 Gy versus 2.5 Gy × 2, and 2.5 Gy × 2 versus 2.5 Gy × 2 + DCA for A549 and H1299 cells were significant (*P<0.05). DCA inhibits DSB repair in A549 and H1299 NSCLC cells (bottom panels). A549 and H1299 cells were plated into groups of T-25 flasks and after 24 h 0 or 50 mM DCA was added to the flasks. After 24 h of 0 or 50 mM DCA treatment, groups of flask of each cell type were irradiated on ice with 20 Gy of X-rays. At various time points from 0.5 to 3 h postirradiation groups of A549 and H1299 cells were taken and prepared for neutral comet assay. The comet olive tail moment of 50 cells from each treatment group and timepoint were measured and the mean and SD plotted (*n=3 experiments, mean ± SD) versus time postirradiation for A549 (top panel) and H1299 (bottom panel) are shown. *t tests indicated that differences in the olive tail moments at these time points postirradiation for A549 and H1299 treated with 0 or 50 mM DCA were significant (*P<0.05).
observed in DCA-treated NSCLC cells. This may be especially relevant in A549 cells with activated K-ras mutations. K-ras mutations have been shown to cause metabolic reprogramming in human cancer cells that result in glucose-6-phosphate being diverted into the nonoxidative shunt of the pentose phosphate pathway [52,53] to synthesize nucleotides. Since we have shown that DCA partially reverse Warburg metabolism in A549 cells, this may indeed reduce available nucleotide pools and slow DNA repair. Future studies will focus on determining the mechanism of DCA X-ray sensitization of NSCLC cells; however, our results strongly suggest that DCA sensitizes NSCLC cells to X-ray-induced cell killing through both altered cell cycle distribution and inhibition of DNA double-strand break repair.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2015.08.006.

References


Supplemental Figure 1S A549 cells (top panel) and H1299 cells (bottom panel) were plated into groups of T25 flasks and after 24 hours were treated by adding 0 up to 50 mM DCA to the flasks. After 48 hours of 0 to 50 mM treatment with DCA, single cell suspensions were prepared and standard colony formation assay to determine percent plating efficiency and surviving fraction.
Supplemental Figure 2S Protein lysates were obtained from A549 and H1299 cells treated with 0 or 50 mM DCA and Western blots were performed for Cyclin D, Cyclin E, cdk4, Cyclin B and pTyr-cdk1 (representative blots are shown in the bottom panels). The western blots from three independent experiments were scanned and the densitometry for each protein band were measured. The mean ± s.d. relative intensity value of each band to its untreated (0mM DCA) control value for A549 and H1299 cells was calculated and are shown above each protein band.
A549 cells were plated into groups of T25 flasks and after 24 hours were treated by adding 0, 17.5, 37.5, or 50 mM DCA to the flasks. After 24 hours of treatment with 0, 17.5, 37.5, or 50 mM DCA, groups of flask from each treatment group were irradiated with 0, 2, 3, or 5 Gy of 160 kVp X-rays and then returned to the incubator for 24 hours of growth. Single cell suspensions were prepared from each experimental group and plated for standard colony formation assay to determine the surviving fraction. Surviving fractions at 0, 2, 3, and 5 Gy for A549 cells treated adding 0, 17.5, 37.5, or 50 mM DCA are shown (n=3 experiments, SF mean ± s.d.). It is important to note that any toxicity from DCA was normalized out in the X-ray survival curves to directly test whether treatment with DCA sensitized the NSCLC cells to X-ray induced cell killing.
Supplemental Figure 4S ATCC2503 immortalized bronchial epithelial cells were plated into groups of T25 flasks and after 24 hours were treated by adding 0 or 50 mM DCA to the flasks. After 24 hours of 0 or 50 mM treatment with DCA, single cell suspensions were: (top left panel) irradiated with 3 Gy and after 24 hours of additional incubation were prepared for standard colony formation assay to determine surviving fraction; (top right panel) irradiated with 2, 3, or 5 Gy of X-rays and after 24 hours of additional incubation were prepared for standard colony formation assay to determine surviving fraction at each X-ray dose; or (bottom panel) after 24 additional hours of 0 or 50 mM treatment with DCA single cell suspensions of ATCC2503 were prepared for DNA flow cytometry analysis after propidium iodide (PI) staining and the percentage of cells in G1, S, and G2/M phase of cells were measured and the average values plus the s.d. for three independent experiments (n=3) are shown (top panels).