



Sodium dichloroacetate (DCA) reduces apoptosis in colorectal tumor hypoxia

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ARTICLE INFO

Article history:

Received 20 January 2010

Received in revised form 6 April 2010

Accepted 15 April 2010

Keywords:

Dichloroacetate

Colorectal cancer

Apoptosis

Hypoxia

Tumor microenvironment

ABSTRACT

We examined the effect of hypoxia on apoptosis of human colorectal cancer (CRC) cells in vitro and in vivo. All cell lines tested were susceptible to hypoxia-induced apoptosis. DCA treatment caused significant apoptosis under normoxia in SW480 and Caco-2 cells, but these cells displayed decreased apoptosis when treated with DCA combined with hypoxia, possibly through HIF-1 α dependent pathways. DCA treatment also induced significantly increased growth of SW480 tumor xenografts, and a decrease in TUNEL positive nuclei in hypoxic but not normoxic regions of treated tumors. Thus DCA is cytoprotective to some CRC cells under hypoxic conditions, highlighting the need for further investigation before DCA can be used as a reliable apoptosis-inducing agent in cancer therapy.

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1. Introduction

Pyruvate dehydrogenase kinase (PDK) inhibits phosphorylation of pyruvate dehydrogenase (PDH) and DCA inhibits the activity of PDK, leading to the activation of the pyruvate dehydrogenase multienzyme complex (PDC) [1]. PDC catalyzes the aerobic metabolism of glucose, pyruvate and lactate, the latter of which is in equilibrium with pyruvate. PDC increases irreversible oxidation of lactate via pyruvate, which then enters the Krebs cycle as acetylCoA and generates NADH and ultimately ATP [1]. The small molecule dichloroacetate (DCA) has been in use for many years to treat diseases such as lactic acidosis and inherited defects in mitochondrial metabolism and is considered relatively low in toxicity [1,2]. Lactic acidosis is also the common state of metabolism in cancer cells, which often have inactivated PDC, and cancer cells generally use glycolysis

rather than respiration (oxidative phosphorylation of glucose) for energy (the Warburg effect) [3–7], possibly as a result of hypoxia that exists in tumors and/or damaged mitochondria [4].

High levels of extracellular lactic acid may contribute to drug resistance [4–8], hence, treatments that reactivate PDC may induce cell death, likely through generation of reactive oxygen species (ROS) and subsequent oxidative damage [2,7,9]. DCA can reprogram mitochondria by reactivating glucose oxidation and has received a great deal of attention for cancer treatment since 2007, when Bonnet et al. showed that exposing rats to 75 mg/l in the drinking water caused regression of their xenografted A549 lung carcinoma cells [8]. Further, in vitro analysis demonstrated that only cancer cells, but not normal somatic cells, were killed by DCA [8], suggesting that DCA could be used as a potent and safe neoadjuvant agent for cancer therapy [9,10]. In addition, DCA significantly sensitized human endometrial cancer cell lines to undergo apoptosis [11], treatment with DCA was associated with decreased rates of cellular proliferation and sensitization to irradiation in prostate cancer cells [12], decreased metastatic breast cancer cell growth in vitro and in vivo [13], and increased cellular oxygen consumption in vitro with increased tumor

Abbreviations: CRC, colorectal cancer; DCA, dichloroacetate; HIF, hypoxia inducible factor.

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hypoxia *in vivo* and led to decreased tumor growth in pancreatic SU86 and colorectal RKO xenografts [14,15]. Based on these reports of anti-cancer activity and the low toxicity of this drug, human clinical trials of DCA for cancer patients are currently planned and/or underway [16]. However, the anti-cancer effect of DCA has only been examined in a limited number of cell lines. In fact, although human colorectal cancer is one of the most prevalent solid tumors in North America, to date, anti-cancer properties of DCA on this type of cancer have not yet been evaluated.

Angiogenesis, the process of new blood vessel formation from pre-existing blood vessels, occurs normally during wound healing, reproduction and fetal development but is also a fundamental step in tumor establishment and growth [17,18]. However, in solid tumors, blood vessels are both structurally and functionally abnormal, with increased permeability, disrupted hierarchical branching and inconsistent flow in compressed or occluded segments [19,20]. The net effect of this abnormality is that regions of solid tumors are transiently and/or chronically exposed to ischemia and reperfusion, leading to hypoxia/anoxia [21], fluctuation in nutrient (especially glucose) levels, acidosis and disruptions in pH, and toxic reactive oxygen species (ROS) generation. These microenvironmental conditions are also known to be mutagenic [22,23]. Thus, solid tumors consist of cells exposed to normoxia and transient and chronic ischemia, the impact of which on effectiveness of anti-cancer therapies is not well understood.

We hypothesized that DCA may have differential effects on hypoxic versus normoxic colorectal cancer cells, and found that while some cell lines are refractory to DCA's effects, most colorectal cancer cells examined actually displayed enhanced survival under hypoxic conditions in the presence of DCA. Consistent with this, DCA treated xenografts showed no anti-tumor effect but instead there was enhanced growth of treated tumors. Our findings suggest that DCA may have differential effects on cancer cell survival depending on the regional microenvironment within treated tumors, which may complicate its usefulness as an adjuvant anti-cancer therapy.

2. Materials and methods

2.1. Cell lines

Human colorectal cancer (CRC) cell lines LS174T, SW480, HCT116, DLD-1, and Caco-2 were obtained from ATCC (Manassas, VA, USA). Primary human dermal fibroblasts were also obtained from ATCC and used as non-carcinogenic control cells.

2.2. *In vitro* exposure to hypoxia and/or DCA

Cells were maintained in standard culture conditions: DMEM (Sigma–Aldrich, Oakville, ON, Canada) cell culture medium, supplemented with 10% heat-inactivated fetal bovine serum, 50 µg/ml gentamicin, and 1 mM sodium pyruvate and cultured at 37 °C in a humidified atmosphere containing 5% CO₂ (“normoxia”). Anoxic conditions (LO)

were achieved using a Modular Incubator Chamber (Billups-Rothenberg Inc., Del Mar, CA, USA) modified to permit continuous flushing of the chamber with a humidified mixture of 95% N₂ and 5% CO₂; the oxygen content in the chamber was less than 0.1% in all anoxia experiments. Confluent monolayers of cells were trypsinized, and 5 × 10⁵ cells were seeded into 6-well plates and incubated under normal cell culture conditions overnight. Thereafter, the plates were assigned to control and anoxia treatment, and exposed to these conditions for 48 h. To investigate the effects of DCA, cells were incubated under normoxia or anoxia in the presence or absence of 10 mM DCA (Sigma–Aldrich) for 48 h, or 48 h plus 48 h recovery in normoxia. For the recovery time all cells received fresh media and the DCA treated cells received an additional treatment of 10 mM DCA. After incubation, cells were trypsinized, pellets were washed twice with PBS, and used for assays described below. Each experiment was performed at least in duplicate and repeated at least three times.

2.3. FACS analysis of apoptosis

Apoptosis was quantified using the annexin V-FITC Apoptosis Detection kit (BioVision Research Products, Mountain View, CA, USA). Briefly, 5 × 10⁵ cells/well were seeded in six-well plates and exposed to DCA plus or minus anoxia as described above. After incubation, cells were resuspended in 500 µl binding buffer and stained with annexin V-FITC and propidium iodide and fluorescence intensity was detected and quantified using a FACScan BD Biosciences with three fluorochrome scanner. In all, 10,000 events were counted for each sample.

2.4. Protein isolation and western blotting for caspase-3

Cell pellets were washed with PBS and resuspended in 0.5 ml of cold fresh lysis buffer [1% Triton X-100, 150 mM NaCl, 0.5 mM MgCl₂, 0.2 mM EGTA, and 50 mM Tris–HCl (pH 7.5), with aprotinin (2 µg/ml), DTT (2 mM), and phenylmethylsulfonyl fluoride (PMSF; 1 mM); all from Sigma–Aldrich]. After vortexing and centrifugation (12,500g at 4 °C for 10 min), the supernatant was aliquoted and stored at –80 °C for future use. 100 µg of total protein from samples were run on a 10% polyacrylamide gel under reducing conditions using SDS–PAGE. Proteins were then transferred to polyvinylidene difluoride (PVDF) membrane which was subsequently incubated in 5% milk diluted in 0.1% Tween 20 in TBS (TBST). The membrane was then probed for chemiluminescent detection for caspase-3 using a rabbit primary antibody (1:1000; Cell Signaling Technologies, Danvers, MA, USA), and a goat anti-rabbit peroxidase-conjugated antibody (1:20,000; Sigma–Aldrich) followed by BM chemiluminescence substrate (Roche Applied Sciences, Laval, QC, Canada). Proteins of cells treated with 10 µg/ml etoposide for 6 h were used as positive controls for caspase-3 cleavage. Band intensity was quantified using densitometry with Image J software (NIH).

2.5. Effect of DCA on HIF-1 α

We determined whether DCA could influence the level of hypoxia inducible factor 1- α (HIF-1 α) stabilized under hypoxic conditions by exposing cells to 150 μ M CoCl₂ with and without 10 mM DCA for 48 h. Control cultures did not receive CoCl₂. After the incubation, protein extraction was performed using a BioVision Nuclear/Cytosol Fractionation Kit (BioVision Research Products). Twenty microgram of the nuclear protein lysates were run on a 7.5% polyacrylamide gel under reducing conditions using SDS–PAGE. Proteins were then transferred to PVDF membrane followed by incubation in 5% milk diluted in TBST. Membrane was then probed for chemiluminescent detection of HIF-1 α using a rabbit anti-HIF-1 α (1:5000; R&D Systems, Minneapolis, MN, USA) and a goat anti-rabbit peroxidase-conjugated antibody (1:20,000; Sigma–Aldrich). Protein loading was normalized using rabbit anti-lamin antibody (1:2000; Cell Signaling Technologies).

2.6. AKT analysis

SW480, LS174T Caco-2, DLD-1 and HCT116 cells were maintained in standard culture conditions and exposed to DCA and anoxia as previously described. After 48 h, cells were washed with PBS and lysed using 50 μ l of cell lysis buffer (Cell Signaling Technologies) supplemented with aprotinin (2 μ g/ml, Sigma–Aldrich), phosphatase inhibitors (Phospho-Stop; Roche Applied Sciences), and PMSF (1 mM; Sigma–Aldrich). Cells were scraped from the plates, incubated on ice for 5 min and transferred to a 1.5 ml tube. After vortexing and centrifugation (12,500g at 4 °C for 10 min), the supernatant was aliquoted and stored at –80 °C for future use. Sixty-five microgram of total protein lysates were run on a 10% polyacrylamide gel under reducing conditions. Protein was then transferred to PVDF membrane, which was subsequently incubated in 5% milk diluted in TBST. The membrane was then probed for chemiluminescent detection using rabbit anti-Phospho-Akt (Ser473) primary antibody (1:1000, Cell Signaling Technologies) and a secondary goat anti-rabbit peroxidase-conjugated antibody (1:10,000; Sigma–Aldrich). Membranes were then stripped, blocked with 5% milk in TBST for 30 min and reprobed with rabbit anti-Akt (pan) monoclonal antibody (1:1000, Cell Signaling Technologies) followed by secondary goat anti-rabbit peroxidase-conjugated antibody (1:10,000; Sigma–Aldrich). Band intensity was quantified using densitometry with Image J software (NIH).

2.7. CRC xenografts

All in vivo procedures were performed according to the guidelines and recommendations of the Canadian Council on Animal Care (CCAC) and approved by the University of Guelph local Animal Care Committee. 2×10^6 SW480 cells and 5×10^6 LS174T cells were subcutaneously implanted (in 100 μ l of 0.1% BSA in PBS) into the right flank of immune deficient RAG1[–] mice [24]. Tumor growth was monitored using Vernier calipers and volume determined by the standard formula (length \times width² \times 0.5) [23]. When the average tumor size passed 35 mm³ (25 days for

SW480 cells and 14 days for LS174T cells), mice were randomly allocated into treatment and control groups. In the SW480 trial there were eight mice in each group, and in the LS174T trial there were four mice in each group. For the treated group, drinking water containing 1 mg/ml of DCA (a dose approximately equivalent to 150 mg/kg/day) for 2 weeks for SW480 injected mice and 9 days for the LS174T injected mice. Water was changed daily and control mice received plain drinking water. At the end of the trial mice were euthanized by CO₂ asphyxia followed by cervical dislocation. Tumors were removed and formalin fixed and paraffin embedded or snap-frozen in cryomatrix (stored at –80 °C) for later sectioning.

2.8. Evaluation of hypoxic and necrotic areas

Six micrometer-thick paraffin sections were deparaffinized and incubated in 3% hydrogen peroxide. Slides were washed and underwent antigen retrieval by sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for 10 min at high in the microwave, and then incubated in DAKO Protein Block (DAKO, Mississauga, ON, Canada) followed by incubation in 5% normal goat serum (Vector Laboratories, Burlington, ON, Canada). Sections were then incubated in rabbit anti-carbonic anhydrase-IX (CA-IX) overnight at 4 °C (1:500, Abcam, Cambridge, MA, USA) followed by a biotinylated goat anti-rabbit secondary antibody (1:500, Vector Laboratories). Slides were then treated with RTU Vectastain Elite, ABC reagent (Vector Laboratories) followed by incubation in DAB. Sections were counterstained with Meyer's hematoxylin (Fisher Scientific, Ottawa, ON, Canada) and images were captured using an Olympus BX61 microscope. To consider changes in tumor volume due to both viable and necrotic regions, we quantified the relative cross-sectional area of SW480 tumors occupied by hypoxic cells as demonstrated by CA-IX immunostaining and necrotic regions as demonstrated by acellular eosinophilic regions in H&E stained sections. Slides were coded and scored in a semiblinded manner, and the areas of hypoxic and necrotic tissue were scored and assessed by ImageScope software (Aperio, Vista, CA, USA). The total hypoxic and necrotic area for each section was divided by the total cross-sectional area to obtain the proportion of hypoxic or necrotic area in each tumor.

2.9. Dual immunofluorescence staining for hypoxia and apoptosis

Six micrometer thick cryosections of SW480 tumors were air-dried at room temperature (RT), fixed in 4% paraformaldehyde for 15 min, blocked and stained by CA-IX as described above. This was followed by goat anti-rabbit Cy3 secondary antibody (1:200) (Sigma–Aldrich) for 30 min at RT. Slides were then stained via TUNEL reaction using the In Situ Cell Death Detection Kit, Fluorescein Kit (Roche Applied Science) and counterstained with DAPI. Images were captured using a Leica Opti-Tech epifluorescence microscope equipped with appropriate excitation and emission filters. Control sections received PBS in place of primary antibodies. The number of TUNEL positive condensed

nuclei and TUNEL positive apoptotic bodies were quantified in hypoxic and normoxic regions of five 40X objective fields for each tumor.

2.10. Statistical analysis

Data are presented as means of several independent measurements \pm SD. Statistical analysis (one way ANOVA followed by Tukey's LSD, or unpaired *t*-test) was used to determine differences between means of cell types for each group of experiments. Significance level for all statistical comparisons was $p \leq 0.05$.

3. Results

3.1. DCA protects some CRC cells from apoptosis in anoxia

When cells were exposed to 10 mM DCA for 48 h, apoptotic rate varied from non-significant (DLD-1, HCT116, and LS174T) to 1.8 and 6.0-fold increase over control for Caco-2 and SW480, respectively (Fig. 1). DCA did not alter apoptosis in the non-cancerous human dermal fibroblast cells. Anoxia induced robust apoptosis in all cell lines. Surprisingly, DLD-1 and HCT116 cells exposed to DCA and anoxia for 48 h had no increase in apoptosis compared to anoxia alone and apoptosis rates were actually decreased for LS174T, SW480 and Caco-2 cells in DCA plus anoxia (Fig. 1A). This apparent protective effect of DCA under hypoxic conditions was more extensive when cells underwent a 48 h recovery in normoxic conditions in the presence of DCA (Fig. 2).

3.2. Caspase-3 activation after DCA and/or anoxia in vitro

Similar to what we observed with annexin V staining, anoxia induced increased caspase-3 fragmentation in all cell lines (Fig. 3A). DLD-1 cells were less affected by anoxia and DCA did not alter the anoxia induced caspase-3 cleavage in these cells. However, combined anoxia and DCA treatment led to reduced caspase-3 fragmentation in Caco-2, LS174T and SW480 cells (Fig. 3A).

3.3. Modulation of HIF-1 α content by DCA

HIF-1 α is the highly labile component of the HIF complex responsible for regulating oxygen responsive gene expression. Since the half-life of HIF-1 α in normoxic cells is approximately 5 min, under normal conditions or upon cellular re-oxygenation after hypoxia, HIF-1 α is completely degraded and can hardly be detected in cells or tissues. Hypoxia, transition metals such as CoCl₂, or the iron chelator desferrioxamine, provoke HIF-1 α stabilization leading to nuclear translocation and promoter bind-

ing. Since re-oxygenation at the end of the experiment could rapidly affect the levels of HIF-1 α before cell lysis was complete, CoCl₂ was used to mimic the anoxic conditions for these studies of HIF levels in cultured cells. As shown in Fig. 3B, treatment of CRC cell lines with 150 μ M CoCl₂ led to nuclear accumulation of HIF-1 α . Although DCA alone did not lead to any changes in HIF-1 α levels, DCA in combination with hypoxia (CoCl₂) decreased the accumulation of HIF-1 α compared to treatment with CoCl₂ alone in all five CRC cell lines (Fig. 3B).

3.4. Changes in AKT activation after DCA exposure

AKT (protein kinase B) was activated by anoxia in DLD-1, Caco-2, and LS174T cells but not in HCT116 cells; SW480 cells did not show any detectable pAKT under these non-stimulated conditions (Fig. 3C), as has been reported previously [25–28]. Interestingly, DCA (in the presence or absence of anoxia) increased phosphorylated AKT compared to control or anoxia in all cell lines except SW480 (Fig. 3C); this response did not correlate with DCA's effects on apoptosis, suggesting other pathways are likely involved in the differential cellular survival we report here.

3.5. Effect of DCA on CRC xenografts

SW480 tumors were significantly larger than their respective control tumors after DCA treatment (Fig. 4A) but there was no significant effect of DCA on LS174T tumor size (Fig. 4B). DCA significantly reduced anoxia-induced apoptosis and CoCl₂-induced HIF-1 α accumulation in LS174T cells (Figs. 1 and 3B). Consistent with this, DCA failed to inhibit LS174T tumor growth (Fig. 4B). To consider both viable and necrotic regions for tumor volume, we quantified the relative cross-sectional area of SW480 tumors occupied by hypoxic cells (as demonstrated by CA-IX immunostaining; arrows in Fig. 4C) and necrotic regions (as demonstrated by acellular eosinophilic regions; asterisks in Fig. 4D) of adjacent sections of the same tumor. There were no significant differences in the relative proportion of tumors occupied by either hypoxic or necrotic tissue between control and DCA treated SW480 xenografts (Table 1).

3.6. TUNEL staining in hypoxic regions

DCA is reported to induce apoptosis in cancer cells but our in vitro results indicated that this is cancer cell type specific and that some cell lines including SW480 are protected from anoxia-induced apoptosis by DCA. Therefore, we used dual immunostaining for CA-IX and TUNEL to quantify apoptosis in hypoxic and normoxic regions in the SW480 xenografts. There were no significant differences in the average number of apoptotic nuclei per field between control and DCA treated tumors (Fig. 5A–C). However, there were significantly fewer TUNEL positive nuclei in hypoxic regions of DCA treated but not control tumors (Fig. 5D). Taken together, these data support the possibility that DCA treatment enhanced SW480 tumor growth by preventing apoptosis in hypoxic regions of tumors, consistent with our in vitro findings.

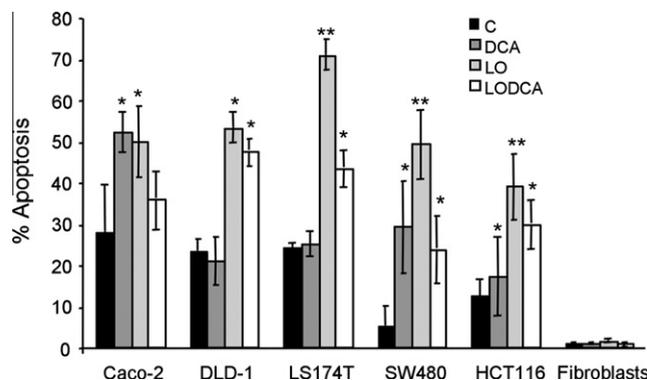


Fig. 1. Average level of apoptosis calculated from three independent experiments. Effect of 10 mM DCA on apoptosis in five CRC cell lines and normal fibroblasts was determined after incubation of the cells under normoxia and anoxia (LO) in the presence or absence of 10 mM DCA for 48 h. The effect of DCA on apoptosis of cancer cells is cell line-dependent and by comparing the anoxia group with the group receiving combination treatment, most cell lines show cytoprotection from anoxic apoptosis in the presence of DCA. * Significantly different from control for that cell line; $p < 0.05$, ** significantly different LO from LODCA for that cell line; $p < 0.05$.

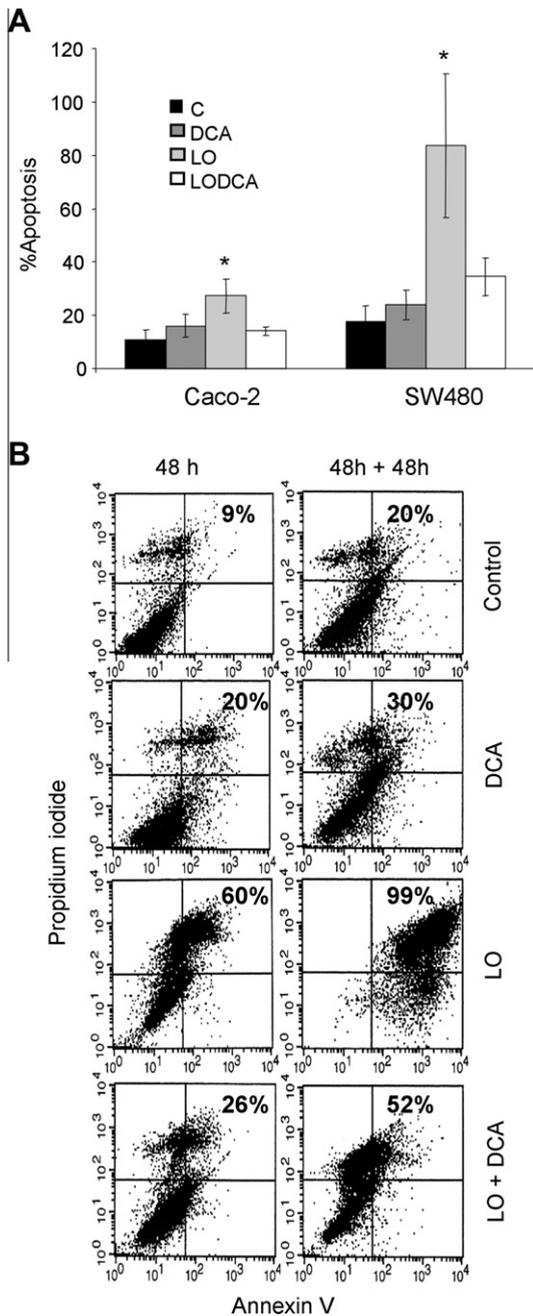


Fig. 2. (A) Average level of apoptosis calculated from three independent flow cytometry experiments. Effect of 10 mM DCA on apoptosis in Caco-2 and SW480 cell lines after incubation of the cells under normoxia and anoxia (LO) in the presence or absence of 10 mM DCA for 48 h plus 48 h recovery in normoxic conditions. * Significant difference from respective control ($p < 0.05$). (B) Analysis of apoptosis in SW480 cells by flow cytometry. The cells were incubated under normoxia and anoxia (LO) in the presence or absence of 10 mM DCA for 48 h, or 48 h plus 48 h recovery in normoxic condition. Percent value indicates the proportion of dead cells.

4. Discussion

In this study, we examined the effect of DCA on apoptosis of human CRC cells and demonstrated that not all cell

lines were susceptible to DCA induced apoptosis, and that DCA could provide a cytoprotective effect under hypoxic conditions. Consistent with this, we report a lack of therapeutic effect (and evidence of tumor promotion) likely due to increased survival under hypoxia in SW480 xenografts treated with DCA. Hypoxia caused by inadequate access to blood vessels and/or their poor perfusion and functionality, plays a role in the development of drug resistance and selection of cancer therapy for solid tumors [17–21]. Recently Anderson et al. [29] reported that DCA could promote proliferation and survival of hypoxic cells. Their finding supports our study, which is the first to examine the interaction between DCA induced apoptosis and tumor microenvironmental conditions such as hypoxia.

Here we confirm that the apoptotic effects of DCA are cancer cell line specific, as has previously been reported. DCA treatment increased PUMA transcripts in endometrial carcinoma cell lines with an apoptotic response, suggesting a p53-PUMA-mediated mechanism may also be involved in sensitizing cancer cell lines to DCA induced apoptosis [11]. Somewhat surprisingly, we found that DCA induced PKB/AKT activation in Caco-2, LS174T, DLD-1, and HCT116 cells, consistent with a pro-survival pathway. Lingohr et al. [30] reported that when mice were administered 0, 0.5, or 1.0 g/l DCA for 10 weeks, DCA decreased their liver PKB expression, although they did not measure levels of phosphorylated PKB/AKT.

Activated phosphorylated AKT (pAKT) has been shown to protect normal and cancer cells against hypoxia and p53-induced apoptosis [31]. The AKT survival pathway is positively and negatively regulated by PI3 K and PTEN, respectively, through their opposing effects on phosphatidylinositol-3-phosphate (PIP3) generation [32] and hypoxia protects PC12 cells from apoptosis through activation of the PI3 K/AKT survival pathway [25]. Interestingly, mitochondrial respiration deficiency leads to activation of the AKT survival pathway through NADH-mediated inactivation of tumor suppressor PTEN [26]. Under hypoxic conditions, cancer cells use the glycolytic pathway to generate ATP, leading to accumulation of high levels of NADH, which is normally channeled to the electron transport chain in respiration-competent cells. The increase in NADH caused by respiratory deficiency inactivates PTEN through a redox modification mechanism, leading to AKT activation [26]. Since DCA inhibits PDK activity and consequently prevents increased NADH production, we expected DCA would reduce anoxia-stimulated activation of AKT. Instead we observed increased p-AKT in DCA treated cells but no significant modulation of this event by anoxia. Further, we saw no correlation between AKT activation by DCA in anoxia and avoidance of anoxia-induced apoptosis, suggesting alternative survival pathways may be involved in the differential cellular responses we report here. Bates et al. [33] have shown that ligation of CD44 can lead to up-regulation of PI3 K/Akt activity and reduced apoptosis. Therefore, a CD44 survival pathway may be involved in the observed AKT activation in our study.

Mitochondrial membrane integrity is disrupted upon apoptotic signaling, leading to cytochrome c release and subsequent activation of the pro-apoptotic caspase cascade [34]. DCA is reported to induce mitochondrial metabolic

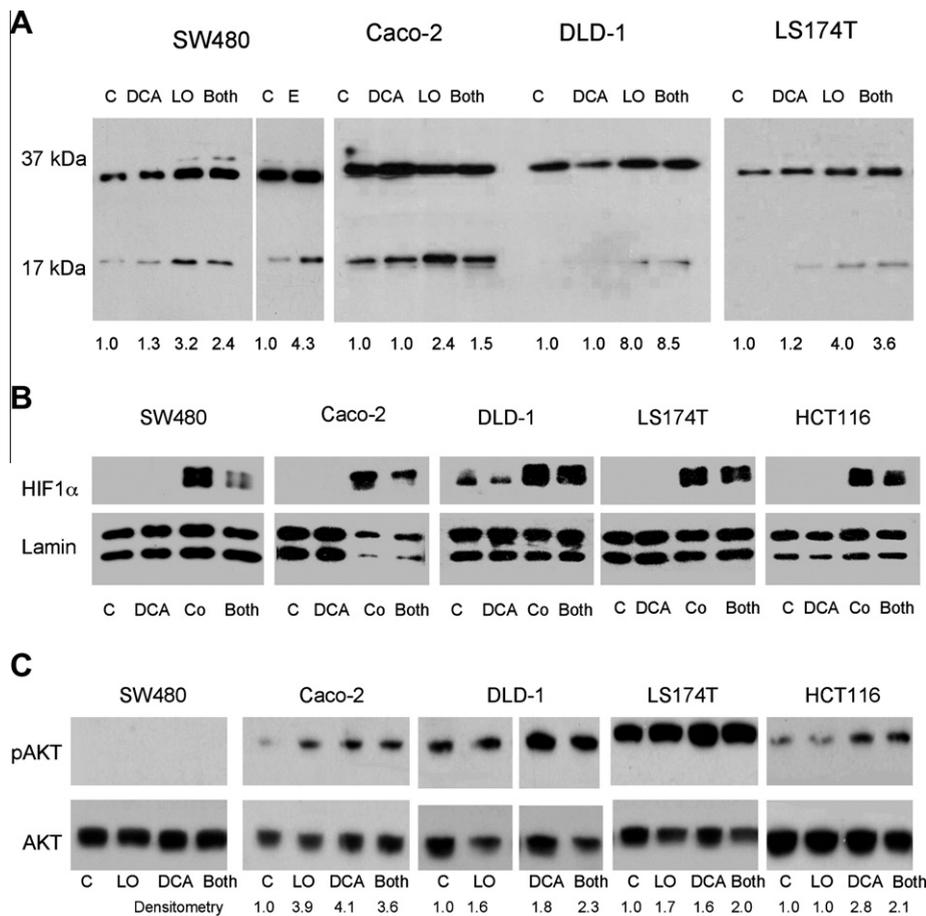


Fig. 3. (A) Caspase-3 western blot analysis of proteins from CRC cells; untreated (C), treated for 48 h with 10 μ M DCA, anoxia (LO) or anoxia plus DCA (Both). Etoposide-treated SW480 cells (E) were used as positive control. Anoxia induced caspase-3 cleavage (17 kDa fragment) in all cell lines, and cells protected from apoptosis by DCA (CaCo-2 and SW480) displayed reduced caspase-3 cleavage under combined conditions compared to anoxia alone; Values are proportion of 17 kDa band to 37 kDa band normalized to control for each cell line. (B) HIF-1 α western blot analysis of nuclear proteins from CRC cell lines; untreated (C), treated for 48 h with 10 mM DCA, 150 μ M CoCl₂ to mimic hypoxia (Co), or CoCl₂ plus DCA (Both); lamin is shown for loading control. As expected, CoCl₂-induced HIF-1 α stabilization in all cell lines. The combination of DCA and CoCl₂ produced reduced amounts of HIF-1 α , but only in cells showing a DCA cytoprotective effect. (C) AKT activation in CRC cells when exposed to LO and/or DCA; untreated (C), treated for 48 h with 10 mM DCA, anoxia (LO), or anoxia plus DCA (Both). DCA in the presence or absence of anoxia led to activation of AKT as demonstrated by phosphorylated protein in all cell lines, regardless of whether they were protected from anoxia-induced apoptosis by DCA; values are proportion of pAKT to AKT normalized to control for each cell line.

changes by remodeling the glycolysis-biased state seen in most cancer cells (due to the Warburg effect) to favour glucose oxidation [8]. However, mitochondria undergoing aerobic glycolysis in favour of glucose oxidation are reported to be relatively resistant to opening of the mitochondrial permeability transition pore and release of cytochrome c. We found that caspase-3 fragmentation in hypoxic cells was decreased by DCA exposure, consistent with a cytoprotective effect mediated through mitochondria that are resistant to DCA induced metabolic switching [8,9].

Treatment with DCA led to decreased apoptosis in hypoxia, compared to anoxia alone, especially in CaCo-2 and SW480 cells. In agreement with these observations, our *in vivo* studies show that SW480 and LS174T xenografts did not respond with growth inhibition when tumor bearing mice were treated with DCA. In fact, SW480 tumors showed significant growth enhancement. Since we found fewer apoptotic cells in hypoxic regions of DCA treat-

ed SW480 compared to normoxic regions DCA modulated inhibition of hypoxia-induced apoptosis apparently occurs both *in vivo* and *in vitro*.

DCA led to decreased HIF-1 α levels under hypoxic but not normoxic conditions. Degradation of HIF-1 α under normoxic conditions is due to hydroxylation at proline 564 and/or proline 402, which is necessary and sufficient for binding to the von Hippel-Lindau protein with concomitant ubiquitination and 26S proteasomal degradation of HIF-1 α [35–37]. Hypoxia or transition metals such as CoCl₂, or the iron chelator desferrioxamine block HIF-PH and stabilize HIF-1 α . DCA binds to the N-terminal domain of PDK and promotes conformational changes leading to the inactivation of kinase activity [38]. PDK, a direct gene target of HIF-1 α , inactivates the enzyme complex PDH, thus attenuating mitochondrial respiration and oxidative phosphorylation. This lack of PDH activity enhances ATP production via increased anaerobic glycolysis, which is

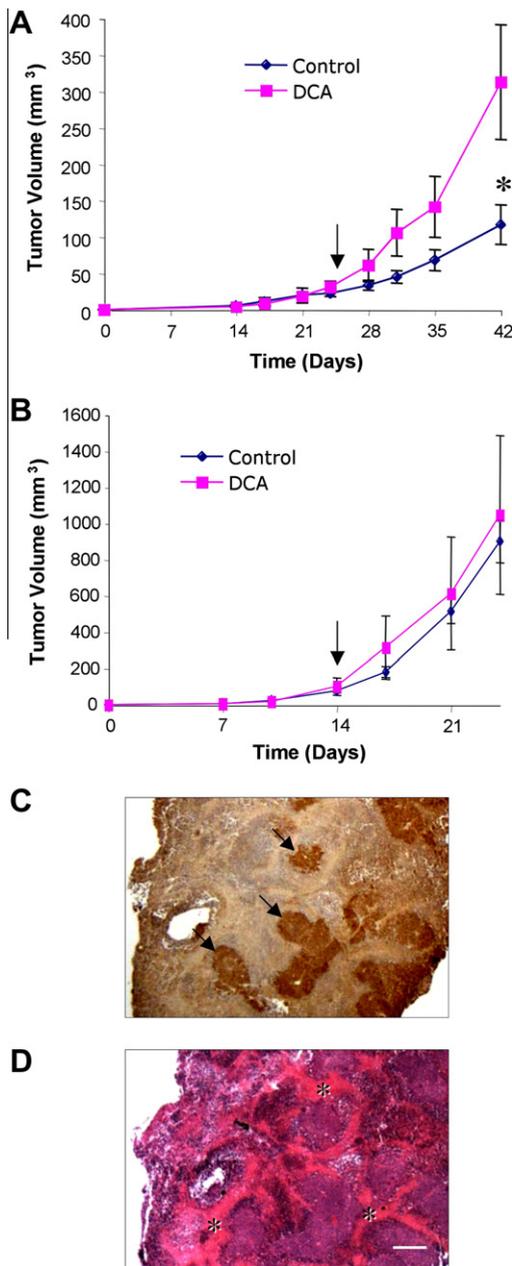


Fig. 4. Effect of DCA on CRC xenograft growth. Two human CRC cell lines (SW480 and LS174T) were xenografted into immune deficient mice. After 25 days for SW480 cells and 14 days for LS174T cells, mice were randomized into treatment and control groups; arrows indicate start of daily DCA treatment. After DCA treatment, SW480 tumors (A) were significantly larger than control ($p = 0.034$) but there was no significant effect of DCA on growth of LS174T tumors (B). Since increased tumor volume could be due to both viable and necrotic regions, the relative cross-sectional areas of SW480 tumors occupied by hypoxic cells (as demonstrated by CA-IX immunostaining; arrows in C) and necrotic regions (as demonstrated by acellular eosinophilic regions; asterisks in D) were quantified in adjacent sections of the same tumor (scale bar = 300 μm). See Table 1 for the results of quantification of C and D.

critical for the adaptation of cells to hypoxia [1,39]. The ability of DCA to decrease HIF-1 α levels in hypoxic cells could lead to reduced PDK production, increased PDH

Table 1

Quantification of hypoxic and necrotic regions in SW480 tumors.

	N	CA-IX (%)	Necrotic (%)
Control	7	44.9 (16.2)	25.7 (12.6)
DCA	6	36.5 (11.8)	29.2 (9.4)

Values are mean (SD). There are no significant differences in the proportion of hypoxic (as CA-IX positive) or necrotic tumor tissue between control and DCA treated tumors ($p > 0.05$).

activity, and a shift in cellular metabolism from glycolysis to glucose oxidation. A shift in metabolism from glycolysis to glucose oxidation is thought to be the main mechanism utilized by DCA to induce selective cancer cell killing, via generation of excessive ROS [8]. However, HIF-1 α also induces synthesis of pro-apoptotic proteins, such as BNIP3 and Noxa [40,41], thus, a reduction in HIF-1 α levels may lead paradoxically to increased cell survival, as has been recently reported with H9c2 cells, where knockdown of HIF-1 α protein via RNAi enhanced cell survival under hypoxic conditions [42]. This finding is consistent with the reduced apoptosis in colorectal cancer cells exposed to DCA and hypoxia/anoxia seen here.

Although there are reports that DCA led to decreased tumor growth in some xenograft models [8,14,15], this present study suggests that there are differences in tumor response with different cells, and that the extent of hypoxic/anoxic regions in tumors may influence DCA's effects. DCA has been in use for many years to treat metabolic conditions and inherited mitochondrial diseases. However, while early case reports and pre-clinical data suggested that DCA might be effective for lactic acidosis, subsequent controlled trials have found no clinical benefit of DCA in that setting [43–45]. This agent is considered relatively non-toxic, however, at 25 mg/kg/day DCA caused clinical peripheral neuropathy [44]. In addition, liver toxicity, neoplasia and skin cancer are induced by DCA in experimental studies [46–48] and some reports suggest that this substance is involved in increased risk of human liver tumorigenesis at high concentrations [49]. The effect of DCA has yet to be evaluated in combination with other cancer therapies, despite its purported usefulness as an anti-cancer adjuvant. Based on these concerns and coupled with our finding that DCA actually provides cytoprotection to cancer cells under hypoxic conditions, we suggest that further investigation of this substance is warranted before introducing it as a safe and effective treatment for cancer patients.

Conflict of interest

The authors declare no conflict of interest for this article.

Role of funding source

The Canadian Cancer Society Research Institute had no involvement in any aspect of this study, aside from operating support for the senior author's research activity.

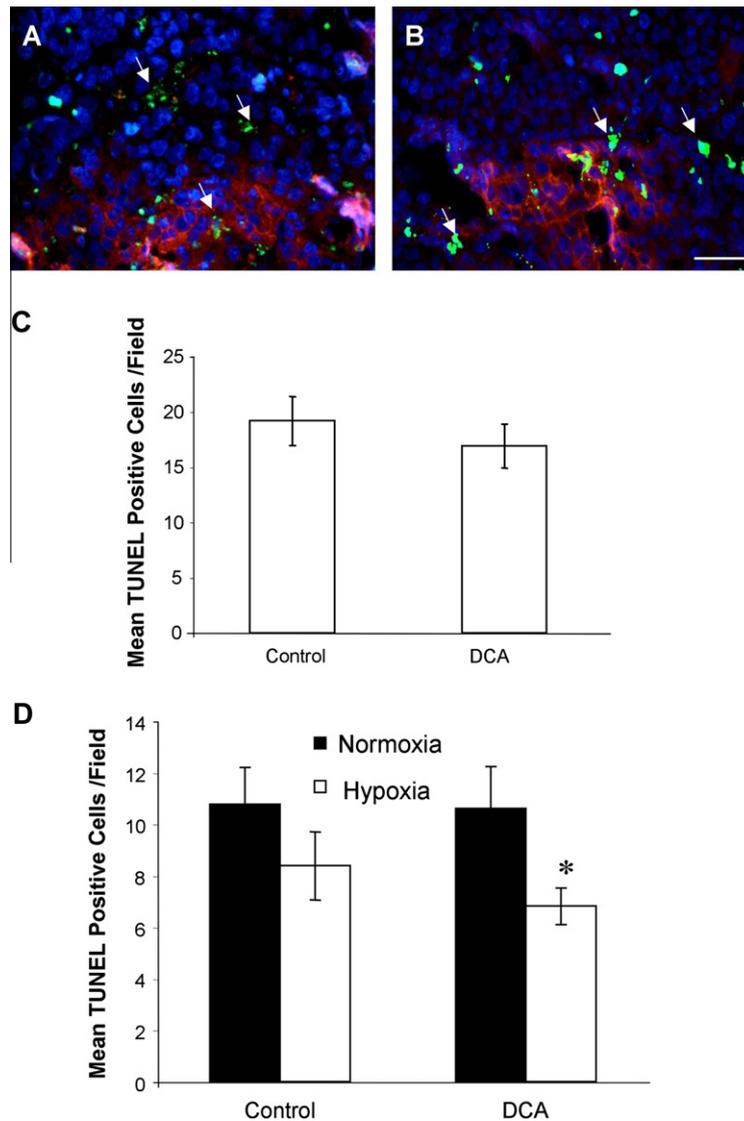


Fig. 5. Quantification of apoptosis in hypoxic and normoxic regions of SW480 xenografts using dual immunostaining for CA-IX (red) and TUNEL (green; arrows) in Control (A) and DCA treated (B) tumors. DAPI counterstain demonstrates nuclei; scale bar = 25 μ m. The number of TUNEL positive nuclei with apoptotic features (TUNEL positive condensed nuclei and TUNEL positive apoptotic bodies) were quantified in five 40X objective fields for each tumor. There were no significant differences in the average number of apoptotic nuclei per field between control and DCA tumors (C) $p > 0.05$. (D) Control tumors had equal numbers of apoptotic nuclei in normoxic and hypoxic regions, but in DCA treated tumors there were significantly fewer apoptotic nuclei in hypoxic regions compared to normoxic regions (* $p = 0.037$). These findings are consistent with the possibility that SW480 cells are relatively protected from apoptosis in hypoxic regions of DCA treated tumors.

Acknowledgments

We are grateful for financial support from the Canadian Cancer Society Research Institute (Grant Number #020094). We would also like to thank Ms. Jackie Rombeek, University of Guelph Central Animal Facility, for assistance with mouse husbandry.

References

- [1] P.W. Stacpoole, N.V. Nagaraja, A.D. Hutson, Efficacy of dichloroacetate as a lactate-lowering drug, *J. Clin. Pharmacol.* 43 (2003) 683–691.
- [2] P.W. Stacpoole, T.L. Kurtz, Z. Han, T. Langae, Role of dichloroacetate in the treatment of genetic mitochondrial diseases, *Adv. Drug Deliv. Rev.* 60 (2008) 1478–1487.
- [3] T. McFate, A. Mohyeldin, H. Lu, J. Thakar, J. Henriques, N.D. Halim, H. Wu, M.J. Schell, T.M. Tsang, O. Teahan, S. Zhou, J.A. Califano, N.H. Jeoung, R.A. Harris, A. Verma, Pyruvate dehydrogenase complex activity controls metabolic and malignant phenotype in cancer cells, *J. Biol. Chem.* 283 (2008) 22700–22708.
- [4] R. Xu, H. Pelicano, Y. Zhou, J. Carew, L. Feng, K. Bhalla, M. Keating, P. Huang, Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia, *Cancer Res.* 65 (2005) 613–621.
- [5] M.V. Berridge, P.M. Herst, A. Lawen, Targeting mitochondrial permeability in cancer drug development, *Mol. Nutr. Food Res.* 53 (2009) 76–86.

- [6] P.L. Pedersen, The cancer cell's "power plants" as promising therapeutic targets: an overview, *J. Bioenergy Biomembr.* 39 (2007) 1–12.
- [7] H. Pelicano, D.S. Martin, R.H. Xu, P. Huang, Glycolysis inhibition for anticancer treatment, *Oncogene* 25 (2006) 4633–4646.
- [8] S. Bonnet, S.L. Archer, J. Allalunis-Turner, A. Haromy, C. Beaulieu, R. Thompson, C.T. Lee, G.D. Lopaschuk, L. Puttagunta, S. Bonnet, G. Harry, K. Hashimoto, C.J. Porter, M.A. Andrade, B. Thebaud, E.D. Michelakis, A mitochondria-K⁺ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth, *Cancer Cell* 11 (2007) 37–51.
- [9] E.D. Michelakis, L. Webster, J.R. Mackey, Dichloroacetate (DCA) as a potential metabolic-targeting therapy for cancer, *Brit. J. Cancer* 99 (2008) 989–994.
- [10] J.G. Pan, T.W. Mak, Metabolic targeting as an anticancer strategy: dawn of a new era? *Sci. STKE Apr* 10 (381) (2007) 14.
- [11] J.Y. Wong, G.S. Huggins, M. Debidda, N.C. Munshi, I. De Vivo, Dichloroacetate induces apoptosis in endometrial cancer cells, *Gynecol. Oncol.* 109 (2008) 394–402.
- [12] W. Cao, S. Yacoub, K.T. Shiverick, K. Namiki, Y. Sakai, S. Porvasnik, C. Urbaneck, C.J. Rosser, Dichloroacetate (DCA) sensitizes both wild-type and over expressing Bcl-2 prostate cancer cells *in vitro* to radiation, *Prostate* 68 (2008) 1223–1231.
- [13] R.C. Sun, M. Fadia, J.E. Dahlstrom, C.R. Parish, P.G. Board, A.C. Blackburn, Reversal of the glycolytic phenotype by dichloroacetate inhibits metastatic breast cancer cell growth *in vitro* and *in vivo*, *Breast Cancer Res. Treat.* 120 (2010) 253–260.
- [14] R.A. Cairns, K.L. Bennewith, E.E. Graves, A.J. Giaccia, D.T. Chang, N.C. Denko, Pharmacologically increased tumor hypoxia can be measured by 18F-Fluoroazomycin arabinoside positron emission tomography and enhances tumor response to hypoxic cytotoxin PR-104, *Clin. Cancer Res.* 15 (2009) 7170–7174.
- [15] Y. Chen, R. Cairns, I. Papandreou, A. Koong, N.C. Denko, Oxygen consumption can regulate the growth of tumors a new perspective on the Warburg effect, *PLoS One* 4 (2009) e7033.
- [16] H. Pearson, Cancer patients opt for unapproved drug, *Nature* 446 (2007) 474–475.
- [17] J. Folkman, Angiogenesis: an organizing principle for drug discovery?, *Nat. Rev. Drug Discov.* 6 (2007) 273–286.
- [18] K.A. Rmali, M.C.A. Puntis, W.G. Jiang, Tumor-associated angiogenesis in human colorectal cancer, *Colorectal Dis.* 9 (2006) 3–14.
- [19] N. Raghunand, R.A. Gatenby, R.J. Gillies, Microenvironmental and cellular consequences of altered blood flow in tumours, *Brit. J. Radiol.* 76 (2003) S11–S22.
- [20] R.K. Jain, Molecular regulation of vessel maturation, *Nat. Med.* 9 (2003) 685–693.
- [21] D.J. Chaplin, R.E. Durand, P.L. Olive, Acute hypoxia in tumors: implications for modifiers of radiation effects, *Int. J. Radiat. Oncol. Biol. Phys.* 12 (1986) 1279–1282.
- [22] E. Papp-Szabo, P.D. Josephy, B.L. Coomber, Microenvironmental influences on mutagenesis in mammary epithelial cells, *Int. J. Cancer* 116 (2005) 679–685.
- [23] S. Shahrzad, L. Quayle, C. Stone, C. Plumb, S. Shirasawa, J.W. Rak, B.L. Coomber, Ischemia-induced K-ras mutations in human colorectal cancer cells: role of microenvironmental regulation of MSH2 expression, *Cancer Res.* 65 (2005) 8134–8141.
- [24] J. Chen, Y. Shinkai, F. Young, F.W. Alt, Probing immune functions in RAG-deficient mice, *Curr. Opin. Immunol.* 6 (1994) 313–319.
- [25] M. Alvarez-Tejado, S. Naranjo-Suarez, C. Jiménez, A.C. Carrera, M.O. Landázuri, L. del Peso, Hypoxia induces the activation of the phosphatidylinositol 3-kinase/Akt cell survival pathway in PC12 cells: protective role in apoptosis, *J. Biol. Chem.* 276 (2001) 22368–22374.
- [26] H. Pelicano, R.H. Xu, M. Du, L. Feng, R. Sasaki, J.S. Carew, Y. Hu, L. Ramdas, L. Hu, M.J. Keating, W. Zhang, W. Plunkett, P. Huang, Mitochondrial respiration defects in cancer cells cause activation of Akt survival pathway through a redox-mediated mechanism, *J. Cell Biol.* 175 (2006) 913–923.
- [27] C. Lechanteur, N. Jacobs, R. Greimers, V. Benoît, V. Derogowski, A. Chariot, M.P. Merville, V. Bours, Low daunomycin concentrations protect colorectal cancer cells from hypoxia-induced apoptosis, *Oncogene* 24 (2005) 788–1793.
- [28] M. Kim, S.Y. Park, H.S. Pai, T.H. Kim, T.R. Billiar, D.W. Seol, Hypoxia inhibits tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by blocking Bax translocation, *Cancer Res.* 64 (2004) 4078–4081.
- [29] K.M. Anderson, J. Jahesh, P. Guinan, M. Rubenstein, *In vitro* effects of dichloroacetate and CO₂ on hypoxic HeLa cells, *Anticancer Res.* 29 (2009) 4579–4588.
- [30] M.K. Lingohr, B.D. Thrall, R.J. Bull, Effects of dichloroacetate (DCA) on serum insulin levels and insulin-controlled signaling proteins in livers of male B6C3F1 mice, *Toxicol. Sci.* 59 (2001) 178–184.
- [31] Y. Ogawara, S. Kishishita, T. Obata, Y. Isazawa, T. Suzuki, K. Tanaka, N. Masuyama, Y. Gotoh, Akt enhances Mdm2-mediated ubiquitination and degradation of p53, *J. Biol. Chem.* 277 (2002) 21843–21850.
- [32] V. Stambolic, A. Suzuki, J.L. de la Pompa, G.M. Brothers, C. Mirtsos, T. Sasaki, J. Ruland, J.M. Penninger, D.P. Siderovski, T.W. Mak, Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN, *Cell* 95 (1998) 29–39.
- [33] R.C. Bates, N.S. Edwards, G.F. Burns, D.E. Fisher, A CD44 survival pathway triggers chemoresistance via lyn kinase and phosphoinositide 3-kinase/Akt in colon carcinoma cells, *Cancer Res.* 61 (2001) 5275–5283.
- [34] I. Samudio, M. Fiegl, M. Andreeff, Mitochondrial uncoupling and the Warburg effect: molecular basis for the reprogramming of cancer cell metabolism, *Cancer Res.* 69 (2009) 2163–2166.
- [35] M. Ivan, K. Kondo, H. Yang, W. Kim, J. Valiando, M. Ohh, A. Salic, J.M. Asara, W.S. Lane, W.G. Kaelin Jr., HIF1α targeted for VHL-mediated destruction by proline hydroxylation: implications for O₂ sensing, *Science* 92 (2001) 464–468.
- [36] P. Jaakkola, D.R. Mole, Y.M. Tian, M.I. Wilson, J. Gielbert, S.J. Gaskell, A.V. Kriegsheim, H.F. Hebestreit, M. Mukherji, C.J. Schofield, P.H. Maxwell, C.W. Pugh, P.J. Ratcliffe, Targeting of HIF-1α to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation, *Science* 292 (2001) 468–472.
- [37] F. Yu, S.B. White, Q. Zhao, F.S. Lee, HIF-1α binding to VHL is regulated by stimulus-sensitive proline hydroxylation, *Proc. Natl. Acad. Sci. USA* 98 (2001) 9630–9635.
- [38] M. Kato, J. Li, J.L. Chuang, D.T. Chuang, Distinct structural mechanisms for inhibition of pyruvate dehydrogenase kinase isoforms by AZD7545 dichloroacetate and radicicol, *Structure* 15 (2007) 992–1004.
- [39] J.W. Kim, I. Tchernyshyov, G.L. Semenza, C.V. Dang, HIF-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia, *Cell Metab.* 3 (2006) 177–185.
- [40] J.Y. Kim, H.J. Ahn, J.H. Ryu, K. Suk, J.H. Park, BH3-only protein Noxa is a mediator of hypoxic cell death induced by hypoxia-inducible factor 1α, *J. Exp. Med.* 199 (2004) 113–124.
- [41] A.E. Greijer, E. van der Wall, The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis, *J. Clin. Pathol.* 57 (2004) 1009–1014.
- [42] R. Malhotra, D.W. Tyson, H.M. Rosevear, F.C. Brosius 3rd, Hypoxia-inducible factor-1α is a critical mediator of hypoxia induced apoptosis in cardiac H9c2 and kidney epithelial HK-2 cells, *BMC Cardiovasc. Disord.* 8 (2008) 9 (Apr 30).
- [43] P. Stacpoole, D. Kerr, C. Barnes, S. Bunch, P. Carney, E. Fennell, N. Felitsyn, R. Gilmore, M. Greer, G. Henderson, A. Hutson, R. Neiberger, R. O'Brien, L. Perkins, R. Quisling, A. Shroads, J. Shuster, J. Silverstein, D. Theriaque, E. Valenstein, Controlled clinical trial of dichloroacetate for treatment of congenital lactic acidosis in children, *Pediatrics* 117 (2006) 1519–1531.
- [44] P. Kaufmann, K. Engelstad, Y. Wei, S. Jhung, M. Sano, D. Shungu, W. Millar, X. Hong, C. Gooch, X. Mao, J. Pascual, M. Hirano, P. Stacpoole, S. DiMauro, D. De Vivo, Dichloroacetate causes toxic neuropathy in MELAS: a randomized controlled clinical trial, *Neurology* 66 (2006) 324–330.
- [45] P.W. Stacpoole, E.M. Harman, S.H. Curry, T.G. Baumgartner, R.I. Misbin, Treatment of lactic acidosis with dichloroacetate, *N. Engl. J. Med.* 309 (1983) 390–396.
- [46] W.H. Tsai, A.B. DeAngelo, Responsiveness of hepatocytes from dichloroacetic acid or phenobarbital treated mice to growth factors in primary culture, *Cancer Lett.* 99 (1996) 177–183.
- [47] D. Armstrong, R.G. Cameron, Comparison of liver cancer and nodules induced in rats by deoxycholic acid diet with or without prior initiation, *Cancer Lett.* 57 (1991) 153–157.
- [48] M. Robinson, R.J. Bull, G.R. Olson, J. Stober, Carcinogenic activity associated with halogenated acetones and acroleins in the mouse skin assay, *Cancer Lett.* 48 (1989) 197–203.
- [49] J.C. Caldwell, N. Keshava, Key issues in the modes of action and effects of trichloroethylene metabolites for liver and kidney tumorigenesis, *Environ. Health Perspect.* 114 (2006) 1457–1463.