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BRIEF REPORT

Reversal of the glycolytic phenotype by dichloroacetate inhibits metastatic breast cancer cell growth in vitro and in vivo

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Abstract The glycolytic phenotype is a widespread phenomenon in solid cancer forms, including breast cancer. Dichloroacetate (DCA) has recently been proposed as a novel and relatively non-toxic anti-cancer agent that can reverse the glycolytic phenotype in cancer cells through the inhibition of pyruvate dehydrogenase kinase. We have examined the effect of DCA against breast cancer cells, including in a highly metastatic in vivo model. The growth of several breast cancer cell lines was found to be inhibited by DCA in vitro. Further examination of 13762 MAT rat mammary adenocarcinoma cells found that reversal of the glycolytic phenotype by DCA correlated with the inhibition of proliferation without any increase in cell death. This was despite a small but significant increase in caspase 3/7 activity, which may sensitize cancer cells to other apoptotic triggers. In vivo, DCA caused a 58% reduction in the number of lung metastases observed macroscopically after injection of 13762 MAT cells into the tail vein of rats ($P = 0.0001$, $n \geq 9$ per group). These results demonstrate that DCA has anti-proliferative properties in addition to pro-apoptotic properties, and can be effective against highly metastatic disease in vivo, highlighting its potential for clinical use.

Keywords Dichloroacetate · Breast cancer · Glycolysis · Metastasis · Animal model

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Introduction

The glycolytic phenotype, often referred to as the Warburg effect, is a widespread phenomenon in the majority of cancer forms where high rates of glucose uptake and glycolysis occur while mitochondrial respiration is repressed, despite the presence of oxygen. This metabolic characteristic is believed to be acquired for the production of ATP during anaerobic tumor evolution; however, evidence is increasingly indicating that the glycolytic phenotype is accompanied by gene expression changes that are intimately linked to tumorigenic processes, such as resistance to apoptosis and increased metastatic potential [1, 2].

The existence of the glycolytic phenotype in breast cancer has been well described. An altered bioenergetic cellular index (BEC) and a profound shift toward an enhanced glycolytic phenotype has been reported in breast cancers compared to paired normal breast tissue biopsies, and correlated with overall and disease-free survival of the patients [3, 4]. The invasiveness of several breast cancer cell lines has been correlated with a higher constitutive level of the transcription factor HIF-1α in normoxic conditions and decreased induction of HIF-1α in hypoxia, as well as higher lactate production [5]. Immunohistochemically, upregulation of two markers of the glycolytic phenotype (glucose transporter GLUT1 and Na^+/H^+ exchanger NHE-1) was observed in microinvasive foci of ductal carcinoma in situ (DCIS), indicating that adaptation

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to hypoxia and acidosis may represent key events in the transition from in situ to invasive breast cancer [6]. Therefore, reversal of the glycolytic phenotype for prevention of metastasis and recurrence of breast cancer is a relevant treatment strategy.

Pyruvate dehydrogenase (PDH) governs the conversion of pyruvate to acetyl Co-A and therefore can control the flow of metabolites from glycolysis to the citric acid cycle and hence the generation of ATP by mitochondria. PDH is regulated by pyruvate dehydrogenase kinase (PDK) that phosphorylates and inactivates PDH [7]. Dichloroacetate (DCA) inhibits PDK and has recently been proposed as a novel and relatively non-toxic anti-cancer agent [8]. DCA has been shown to reverse the glycolytic phenotype in a number of cancer cell lines, depolarizing the hyperpolarized inner mitochondrial membrane potential to normal levels and increasing mitochondrial metabolism [8, 9]. Because DCA targets a change undergone during tumorigenesis, it can be effective against cancer cells without toxicity to normal cells. DCA is currently in phase III clinical trials for the treatment of chronic lactic acidosis in congenital mitochondrial disorders [10, 11], and thus has the potential to move quickly into the clinic for other applications as it has passed phase I/II toxicity testing in humans [12]. Clinical trials evaluating its toxicity in cancer patients are underway (http://www.clinicaltrials.gov); however, controlled experiments to understand the anti-cancer activities of DCA are needed to determine which tumors and which patients are most appropriate to treat with DCA.

In this study, a rat mammary adenocarcinoma cell line was used to examine the effect of DCA both in vitro and in vivo. The results suggest a mechanism of action for DCA in these cells as an anti-proliferative agent rather than an apoptosis-inducing agent, and demonstrate that DCA can be effective in vivo at reducing metastatic cancer cell growth, increasing its relevance to breast cancer treatment.

Materials and methods

Cell culture

13762 MAT rat mammary adenocarcinoma cells (MAT cells) were maintained in vitro as previously described [13]. V14 cells were derived from a spontaneous mammary adenocarcinoma arising in a BALB/c-*Trp*53$^{+/-}$ mouse [14].

Cell growth

Cells were exposed to 1–5 mM DCA (Sigma Chemical Co St. Louis, MO) for 1–4 days in 96-well plates, with daily renewal of media and DCA, and cell viability was measured by neutral red uptake [15].

Apoptosis

Caspase 3/7 activities in MAT cells were assessed by using Caspase-Glo 3/7 assay (Promega Corp., Madison, WI) according to the manufacturer's instructions. Apoptosis was quantitated by flow cytometry after staining cells with FITC-labeled Annexin-V (BD Pharmingen, NJ) and propidium iodide (PI) (Sigma Chemical Co St. Louis, MO).

Proliferation

Cells were stained with 5 μM carboxyfluorescein succinimidyl ester (CFSE) and examined by fluorescence-activated cell-sorting analysis [16].

Cell metabolism

Internal ATP levels in MAT cells were assessed using CellTiter-Glo assay (Promega Corp., Madison, WI) according to the manufacturer's instructions. Extracellular lactate levels were determined spectrophotometrically by measuring the conversion of NAD to NADH at 340 nm by lactate dehydrogenase in neutralized perchloric acid extracts of media [17].

13762 MAT cell metastasis in vivo

Animal experiments were conducted with the approval of the Australian National University Animal Ethics Experimentation Committee under the guidelines established by the Australian National Health and Medical Research Committee. Three groups of female Fischer 344 rats (10–13 weeks of age) were injected with 2×10^5 13762 MAT cells into the lateral tail vein [13]. Group 1 (control) rats were untreated. Prior to the injection, groups 2 (low dose) and 3 (high dose) received DCA administered orally in drinking water at 0.2 g/l (23 mg/kg) for 7 days to deplete GSTZ1 activity and maximize DCA bioavailability [18]. On the day of cell injection the oral dose was increased to 0.75 g/l (average water consumption 115 ml/kg/day) corresponding to a daily dose of 86 mg/kg without significantly altering water consumption (control 120 ml/kg/day). Rats in group 3 underwent additional DCA treatment (high dose), receiving 200 mg/kg/day intraperitoneally (i.p.) in phosphate buffered saline (neutralized and filter sterilized), with the first injection administered approximately 2 h before cell injection. Extrapolating from published data [18, 19], the oral dosing is estimated to result in plasma concentrations in the range of 0.5–1 mM DCA, while the

additional 200 mg/kg i.p. is estimated to increase this approximately threefold to 1.5–3.0 mM.

Rats were killed 14 days after tumor cell injection, and the lungs were fixed in Bouins solution. The number of lung metastases was assessed under a dissecting microscope. The largest two lobes from each lung were subsequently paraffin-embedded and then stained for microscopic assessment. The number of microscopic lesions, their size, and number of mitoses per high power field (hpf) were assessed. The presence or absence of tumoral necrosis was reported and the density of tumor-associated lymphocytes (occasional/mild/moderate/severe) was recorded.

GSTZ activity

DCA is metabolized to glyoxylate by the glutathione transferase GSTZ1-1 within the liver, but DCA can inhibit its own catabolism by forming an inactive enzyme-substrate complex with GSTZ1 [20]. In order to ensure effective delivery of DCA, GSTZ1 activity was measured in rat liver according to the method previously described [21].

Statistical analysis

FACS data were acquired using the Cell Quest software package (BD Bioscience, Rockville, MD), and were analyzed using FlowJo (Tree Star Inc, OR). Calculations were performed using the GraphPad Prism® software package, and Student's *t*-test was applied to assess differences between DCA-treated and control groups. A P value of less than 0.05 was considered to be statistically significant. Data are represented as mean ± standard deviation.

Results

DCA inhibits breast cancer cell growth

In order to investigate the sensitivity of breast cancer cells to DCA, we treated a panel of breast cancer cell lines with 5 mM DCA (Fig. 1a). MCF-7, T-47D, 13762 MAT and V14 cells all showed a 60–80% decrease in cell number on day 4 of treatment, whereas 4T1 cells were insensitive. In contrast, DCA had no effect on the growth of a non-cancerous control cell line, MCF-10A.

The effect of DCA on MAT cells was examined further both in vitro and in vivo. The response of MAT cells toward DCA was both time- and dose-dependent (Fig. 1b, c). The strongest effect was seen on day 4 when MAT cells treated with 5 mM DCA had 68 ± 5% less cells than the control cultures ($n = 3$, $P < 0.0001$). In order to determine the reason for decreased cell numbers, cell proliferation and apoptosis were measured. Using the CFSE cell proliferation assay, it was found that after 3 days, MAT cells treated with 5 mM DCA showed significantly higher fluorescence compared to the untreated cells ($P = 0.0009$; $n = 3$), indicating reduced cell division (Fig. 2a). This was evident

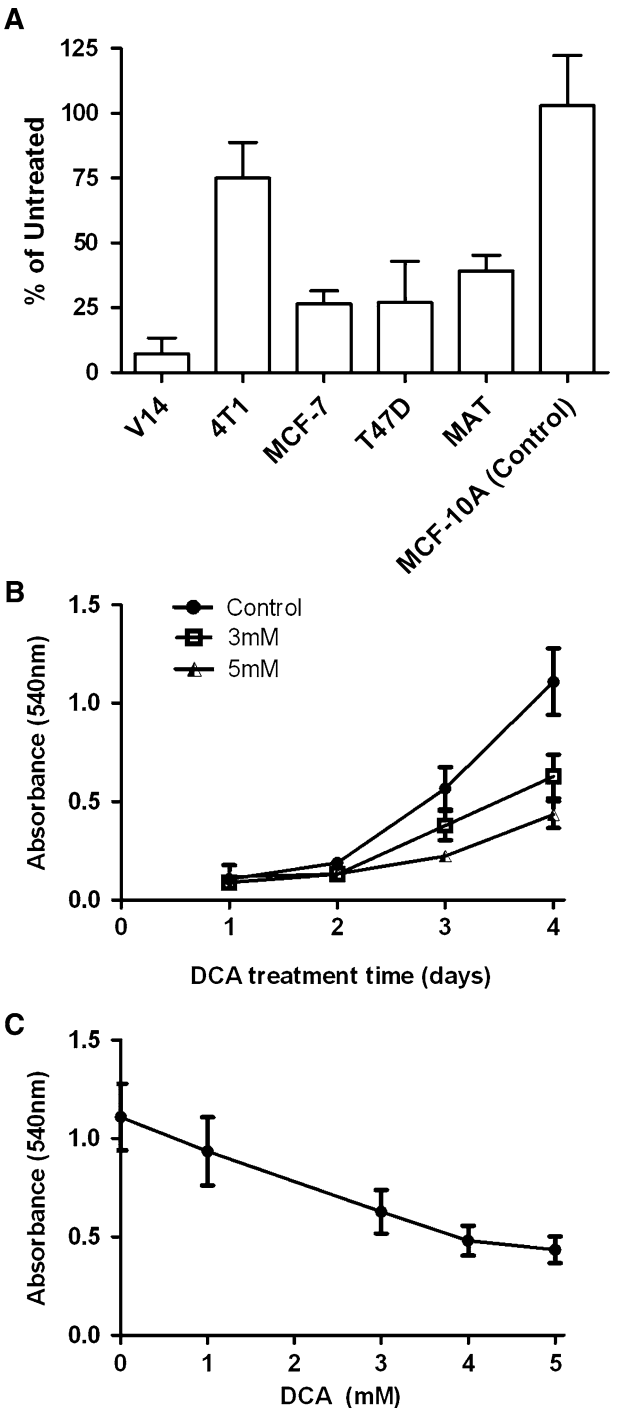

Fig. 1 Effect of DCA on cell growth. **a** A panel of breast cancer cell lines showing different sensitivities toward 4 days of 5 mM DCA treatment. MCF-10A is a non-cancerous control. **b** Time course of inhibition of MAT cell growth by DCA. **c** Dose response of MAT cells toward DCA on day 4 of DCA treatment

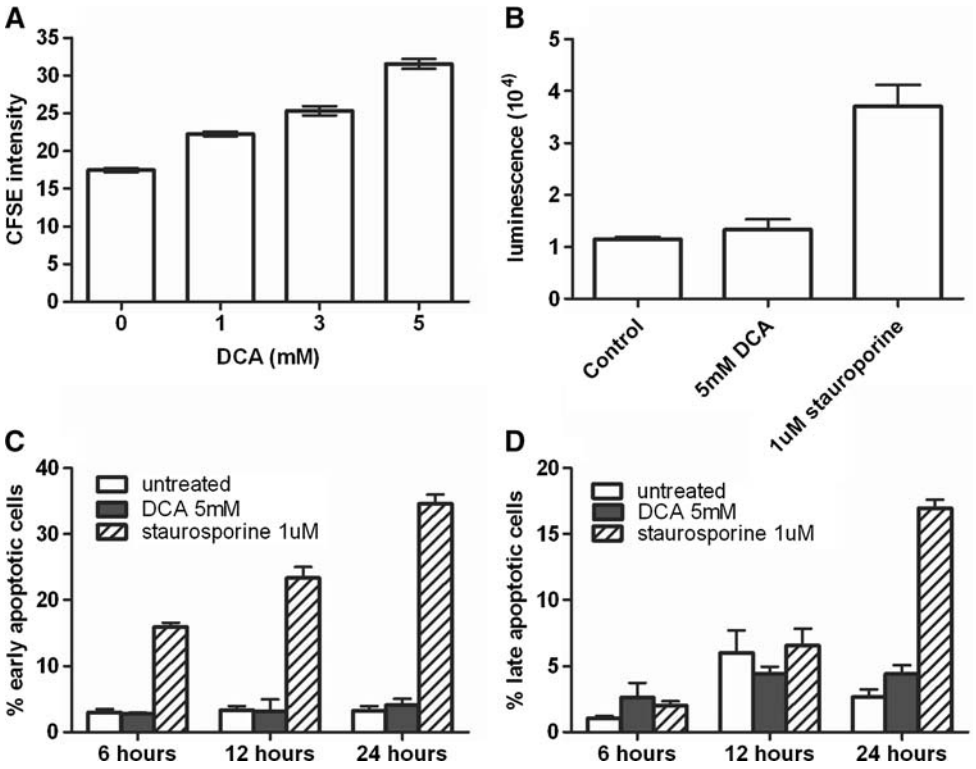

Fig. 2 Effect of DCA on cell proliferation **a** and apoptosis **b–d** indicators in MAT cells. **a** Mean CFSE cell fluorescence after 3 days of DCA treatment. **b** Caspase 3/7 activities after 3 h of treatment. Percentage of early **c** and late **d** apoptotic cells. Staurosporine is a positive control for induction of apoptosis

even at 1 mM DCA. In contrast, apoptosis was not increased by DCA (Fig. 2b–d). Treatment with 5 mM DCA showed a small (15%) but statistically significant increase in caspase 3/7 activity after 3 h (Fig. 2b); however, this was a minimal increase compared to the staurosporine positive control (2.2-fold). Annexin V and PI staining also indicated that 5 mM DCA treatment failed to induce apoptosis in MAT cells even after 24 h incubation (Fig. 2c, d).

DCA reverses the glycolytic phenotype

Treatment of MAT cells with 5 mM DCA for 30 min resulted in a 18 ± 3% increase in total ATP levels ($n = 3$, $P = 0.009$), and this effect persisted at 3 h. After 12 h of DCA treatment, extracellular lactate concentration was decreased by 16.3 ± 5.3% ($n = 4$, $P = 0.01$). These data confirm the reversal of the glycolytic phenotype of MAT cells by DCA.

DCA reduced tumor growth in vivo

After i.v. injection of MAT cells, there was no change in the number of metastases in rats in the low dose DCA group (receiving ~86 mg/kg DCA by the oral route alone). In contrast, rats in the high dose DCA group (receiving daily i.p injections of 200 mg/kg/day DCA in addition to the oral DCA) showed a significant 58 ± 17% reduction in the number of lung tumors observed macroscopically ($P = 0.0001$, $n \geq 9$ per group) (Fig. 3). Microscopically, however, the number of lesions was unchanged across the three groups (6.4 ± 2.8, 7.1 ± 3.4, and 6.2 ± 3.2 per 5 high power fields for control, low, and high dose, respectively). The lesions in the high dose DCA-treated rats developed less tumoral necrosis and had a higher mitotic count (9.4 ± 7.0 vs 20.2 ± 9.2 per 5 hpf in control versus high dose DCA, respectively, $P = 0.03$) (Fig. 4). Apoptotic bodies were not a feature in any of the groups. DCA treatment also led to a moderate lymphocytic infiltration especially at the edges of the tumors whereas the control group was only associated with occasional tumor-associated lymphocytes (Fig. 4).

GSTZ1-1 activity was measured in the liver of DCA-treated, tumor-bearing rats to confirm the exposure of rats to DCA. The low and high dose treatment groups showed a 93% and 95% decrease, respectively in liver GSTZ1-1 activity after DCA treatment, indicating almost complete removal of GSTZ1-1 activity at both doses.

Discussion

The glycolytic phenotype occurs almost universally in breast carcinomas and is associated with microinvasive foci and poorer survival outcomes [2–5]. DCA is an inhibitor of PDK and is able to reverse the glycolytic

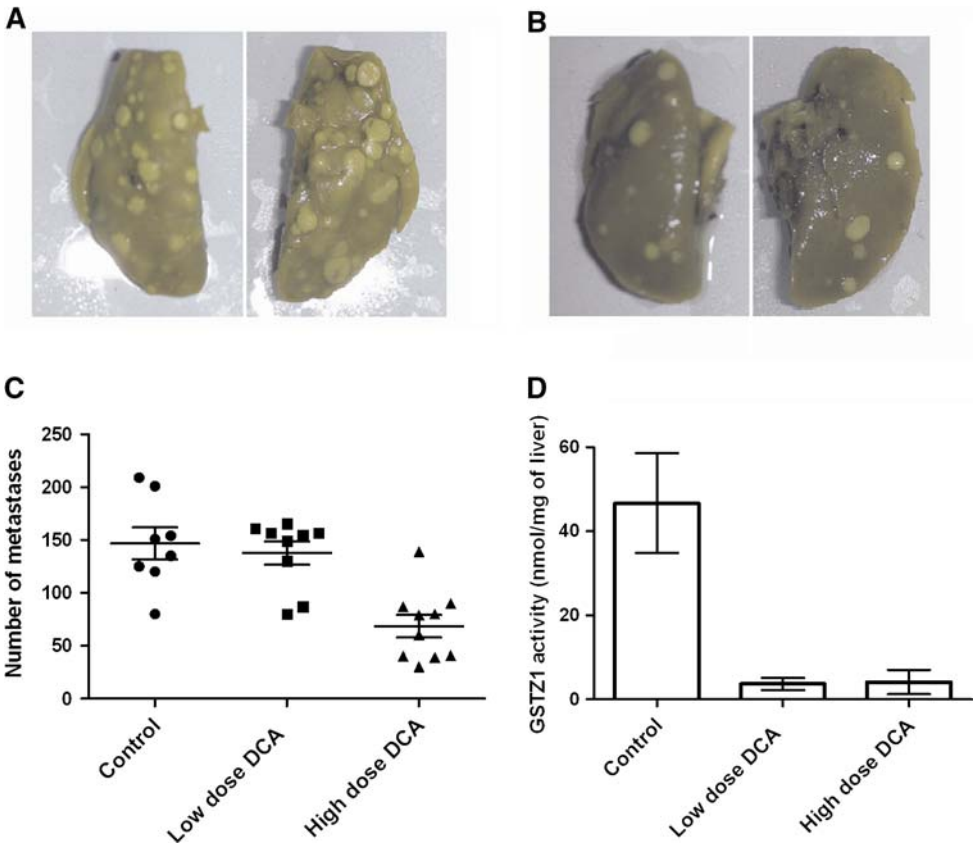

Fig. 3 In vivo studies. Rat lungs showing the metastases formed in control **a** and high dose DCA-treated **b** Fischer rats after tail vein injection of MAT cells. **c** Lung metastases per rat for the control, low and high dose DCA treatment groups. **d** Liver GSTZ1 activity in the same rats

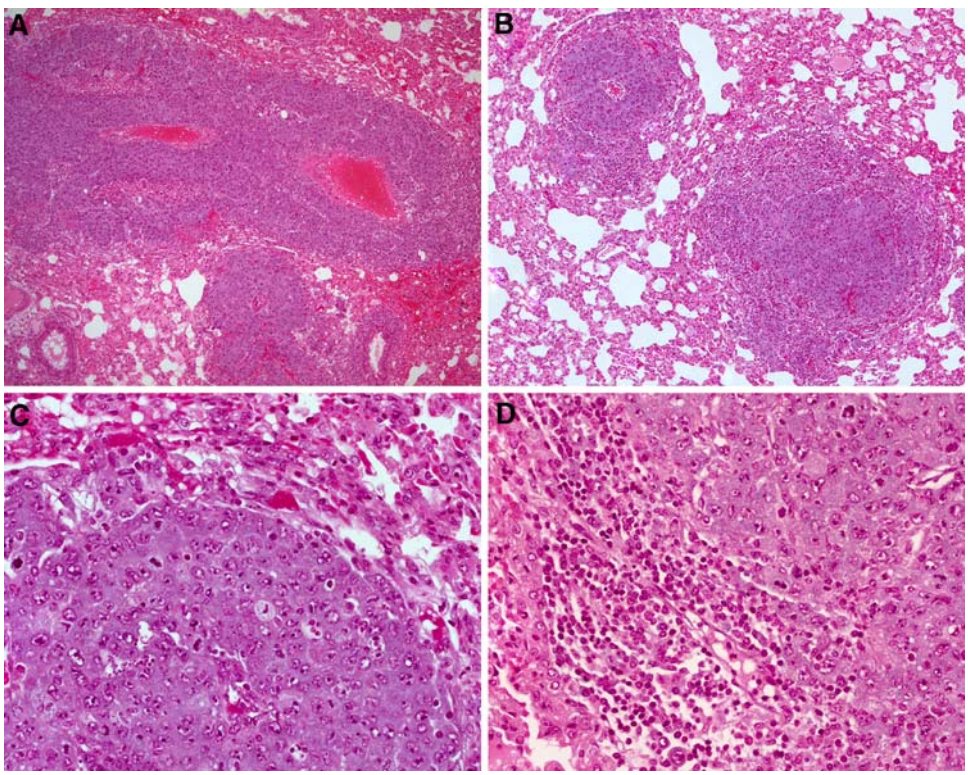

Fig. 4 Photomicrographs showing **a** central necrosis and **c** the lack of a lymphocytic infiltrate in a control rat. In high dose DCA-treated rats, tumors **b** developed less necrosis, and **d** were associated with a moderate lymphocytic infiltrate. (H and E stained, original magnification (**a**, **b**) ×100, (**c**, **d**) ×400)

phenotype. In this study, we report that a number of breast cancer cell lines are sensitive to DCA, with growth inhibition being observed over several days of treatment (Fig. 1). In vitro studies on MAT cells clearly demonstrated that this was due to inhibition of proliferation, with no signs of apoptosis or cell death (Fig. 2). This contrasts with studies published to date on DCA treatment of endometrial, prostate, and lung cancer cells where in most cases, increased apoptosis with no effect on cell cycle distribution [9, 22], or increased apoptosis accompanied by decreased proliferation [8] was reported. Only two out of the six cell lines reported on showed any change in cell cycle distribution after DCA treatment, one with G0/G1 arrest and the other one with some S and some G2/M arrest [9, 22]. While DCA inhibits cell growth in a wide range of cancer cells, the mechanism appears to be cell line dependent. A lack of apoptosis in the human breast cancer cell line T-47D after DCA treatment was also observed (data not shown), indicating that this response is not unique to MAT cells, but may be a characteristic of breast cancer cells. This is an intriguing possibility and will be the subject of further investigation. Alternatively, the lack of apoptosis may be due to high levels of cell survival proteins, such as Bcl-2, survivin, and PUMA, in the particular cell lines tested. The expression of these survival factors was reduced by DCA treatment in prostate, lung, and endometrial cancer cells [8, 22], and this may contribute to the apoptotic response observed.

The sensitivity of breast cancer cell lines to DCA ranged from 20 to 80% inhibition of cell growth over 4 days of 5 mM treatment (Fig. 1), with 4T1 cells being the least sensitive. Sensitivity to DCA may be determined by multiple factors, including the ability to metabolize DCA via GSTZ1, or overexpression of different PDK isoforms. There are four isoforms of PDK, with Kis for DCA of 1.0, 0.2, 8.0, and 0.5 mM, respectively [23]. While PDK1, 2, and 4 would be inhibited by the concentrations of DCA used in this study, PDK3 would not. Expression of PDK3 is normally restricted to testes [23], although expression and induction by hypoxia of PDK3 have been reported for several cancer cell lines [24]. Studies to correlate PDK expression with DCA sensitivity and determine which tumors are most effectively targeted by DCA are underway.

A small increase in caspase 3/7 activities was also seen. This may likely be due to the reactivation of the electron transport chain by DCA and elevation of reactive oxygen and nitrogen species production in the mitochondria [25]. This increase in the basal level of caspase activity may not be sufficient to induce apoptosis; however, it may indicate that DCA could be used to sensitize cancer cells toward other apoptotic triggers, such as hypoxia, radiation, or other chemotherapeutic agents. These potential synergies require further analysis.

In vivo, reversal of the glycolytic phenotype by targeting the pyruvate to acetyl CoA coupling of glycolysis and mitochondrial respiration has previously been demonstrated to be effective against primary tumor growth in two models [1, 8]. We have demonstrated the potential of DCA to be effective against metastatic disease in vivo in the MAT cell model. While the macroscopic number of lung lesions was reduced by high dose DCA, the number of microscopic lesions was not changed, suggesting that the major effect of DCA was on the size of the tumors, rather than a reduction in the number of cells able to establish themselves as tumors in the lungs. The observation of a lower incidence of necrosis in DCA-treated tumors is also consistent with a growth inhibition mechanism as was observed in vitro. The increased number of mitoses appears initially to conflict with this, suggesting higher proliferation rates; however, we suggest this may be due to cell cycle arrest by DCA prior to anaphase, leading to an accumulation of cells present as mitotic figures. Interestingly, there was an increase in tumor-associated lymphocytes in the high dose DCA-treated animals. A stronger immune response against the tumors may be promoted by a reduction in tumor lactate levels achieved by DCA treatment, as high lactic acid concentrations have been shown to reduce T cell function [26]. Experiments with V14 cells in vivo suggest that V14 cells are sensitive to DCA in vivo in a manner similar to the MAT cells, with reduced primary tumor growth and increased lymphocyte presence being observed (data not shown), indicating that these in vivo effects are not unique to MAT cells.

The use of agents at millimolar concentrations is often considered untenable. However, millimolar serum levels of DCA (0.3–1 mM) have been maintained chronically in patients by oral administration of DCA at 25 mg/kg/day [27]. In acute treatment of patients, as much as 80 mg/kg i.v. has been tolerated during liver transplantation [28]. While the effective DCA dose in vivo in this rat model was high, the estimated plasma concentration achieved in vivo (1.5–3 mM, see Methods) is similar to that in humans receiving 25 mg/kg/day, and so is relevant to the clinical setting. This plasma concentration range also correlates with the inhibition of proliferation in vitro (as low as 1 mM, Fig. 2a), and with the Ki for inhibition of PDKs by DCA [23], supporting the proposal that PDK is the target responsible for the anti-cancer activities of DCA.

While chronic DCA treatment in MELAS patients resulted in some reversible peripheral neurotoxicity [10], the toxicity of DCA to tissues vulnerable to classical cytotoxic agents is minimal [8, 29], making it a good candidate agent for combination therapy. For example, while DCA irreversibly inhibits GSTZ1 (Fig. 2d), DCA-treated mice have not shown the lymphocyte depletion observed in mice genetically deficient in GSTZ [21, 30].

Therefore, reversal of the glycolytic phenotype in breast cancer via inhibition of PDK with DCA is a promising anti-cancer strategy and also demonstrates a potential application for alternative PDK inhibitors currently in the drug development pipeline [31]. Nonetheless, further mechanistic studies to understand the causal relationship between the glycolytic phenotype and tumor characteristics are required to enable proficient targeting of cancer cell metabolism for therapeutic purposes.

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