

Novel molecular mechanisms of antitumor action of dichloroacetate against T cell lymphoma: Implication of altered glucose metabolism, pH homeostasis and cell survival regulation

Ajay Kumar, Shiva Kant, Sukh Mahendra Singh *

School of Biotechnology, Banaras Hindu University, Varanasi 221005, India

ARTICLE INFO

Article history:

Received 20 March 2012

Received in revised form 5 June 2012

Accepted 7 June 2012

Available online 15 June 2012

Keywords:

Altered glucose metabolism

Apoptosis

Cytokines

Dichloroacetate (DCA)

pH homeostasis

Reactive oxygen species (ROS)

ABSTRACT

Pyruvate dehydrogenase kinase (PDK) inhibits pyruvate dehydrogenase (PDH) activity and thus promotes energetic switch from mitochondrial glucose oxidation to cytoplasmic glycolysis in cancerous cells (a phenomenon known as the 'Warburg effect') for their energy need, which facilitates the cancer progression by resisting induction of apoptosis and promoting tumor metastasis. Thus, in the present investigation, we explored the molecular mechanisms of the tumoricidal action of dichloroacetate (DCA), a pyruvate dehydrogenase kinase inhibitor, on cells of a murine T cell lymphoma, designated as Dalton's lymphoma (DL). *In vitro* treatment of tumor cells with DCA inhibited their survival accompanied by a modulation of the biophysical composition of tumor-conditioned medium with respect to pH, glucose and lactate. DCA treatment also altered expression of HIF1- α and pH regulators: VAPase and MCT1 and production of cytokines: IL-10, IL-6 and IFN- γ . Moreover, we also observed an alteration in the expression of other apoptosis and cell survival regulatory molecules: PUMA, GLUT1, Bcl2, p53, CAD, caspase-3 and HSP70. The study discusses the role of novel molecular mechanisms underlying DCA-dependent inhibition of tumor cell survival. This study shows for the first time that DCA-dependent alteration of tumor cell survival involves altered pH homeostasis and glucose metabolism. Thus, these findings will provide a new insight for therapeutic applications of DCA as a novel antineoplastic agent against T cell lymphoma.

© 2012 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Neoplastic cells mainly depend on the aerobic glycolysis, known as the 'Warburg effect', for their energy requirement owing to multiple adaptive mechanisms [1,2]. Due to such physiological state tumor cells display a high rate of glycolysis and produce lactate even in the presence of oxygen, which renders growth advantages by resisting induction of apoptosis and facilitating tumor metastasis [1,3].

The energetic switch from mitochondrial glucose oxidation to cytoplasmic glycolysis in cancerous cells is a result of overexpres-

sion of pyruvate dehydrogenase kinase (PDK), which inhibits pyruvate dehydrogenase activity [4]. Pyruvate dehydrogenase (PDH) acts as a gate-keeper enzyme for the entry of pyruvate produced by glycolysis into the mitochondrial tricarboxylic acid (TCA) cycle by converting it to acetyl-CoA and thus plays a crucial role in regulation of cellular metabolism [3,5]. The activity of PDH is mainly regulated by the PDK and pyruvate dehydrogenase phosphatase (PDP), which inactivates and reactivates it respectively. Thus, targeting the aerobic glycolysis is considered to be a promising novel approach for cancer treatment.

Dichloroacetate (DCA), an orally active small molecule is a metabolic modulator that acts as a PDH activator by inhibiting PDK [4–6]. Activation of PDH shifts cancer cell metabolism from glycolysis to glucose oxidation and thus decreases mitochondrial membrane potential, lactate production, augments reactive oxygen species, associated with induction of apoptosis and declined tumor cell proliferation without any harmful manifestation in normal cells [4,5,7–9].

However, the molecular mechanisms of the action of DCA still remain speculative to a large extent. DCA can inhibit the tumor progression in following ways (i) by enhancing apoptosis in endometrial cancer without any effect on cell proliferation [9];

Abbreviations: Bcl-2, B-cell lymphoma 2; CAD, Caspase-activated DNase; DCA, Dichloroacetate; DCFDA, Dichlorodihydrofluorescein diacetate; DL, Dalton's lymphoma; HSP70, Heat shock protein 70; HIF1- α , Hypoxia-inducible factor 1 alpha; IFN- γ , Interferon gamma; IL, Interleukin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PDH, Pyruvate dehydrogenase; PDK, Pyruvate dehydrogenase kinase; GLUT1, Glucose transporter 1; MCT1, Monocarboxylate transporter 1; PUMA, p53 upregulated modulator of apoptosis; ROS, Reactive oxygen species; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; VAPase, Vacuolar-type H⁺-ATPase.

* Corresponding author. Tel.: +91 542 2368331; fax: +91 542 2368693.

E-mail address: sukhmahendrasingh@yahoo.com (S.M. Singh).

(ii) by inhibiting cell proliferation in cancers like breast, lung and prostate with no effect on apoptosis or cell death [6,10]; and (iii) by inducing apoptosis as well as inhibiting proliferation in cancers like colorectal and lung [7,5]. Thus, the mechanisms by which DCA inhibits growth of a variety of cancer cells could vary depending on their etiology.

Considering the fact that about 18% of malignancies are of hematological origin [11], which are also considered complicated for clinical management [12,13], using a murine model of a transplantable T cell lymphoma of spontaneous origin, designated as Dalton's lymphoma (DL), we have been investigating various aspects of host–tumor interactions related to tumor progression and development of effective therapeutic strategies against tumor cells of lymphocyte origin [14–19]. DL originated in DBA (H-2^d) strain of mouse as a thymoma [20,21]. The tumor cells can be grown both as ascites and solid tumor [22], display a massive aneuploidy [23] and gender dimorphism [16]. Moreover, the DL cells show a stage dependent variation in susceptibility to anticancer drugs [17].

Although due to its easy availability and low cost, DCA is considered as a potential drug for antineoplastic applications, it is yet to be approved for anticancer treatment. Nevertheless, it has only been tested against a few types of cancer cells. Since, there are no reports with respect to antineoplastic action of DCA against T cell tumors, this study was undertaken to investigate the so far unexplored molecular mechanisms regarding the antineoplastic action of DCA with respect to hematological malignancy of T cell origin using above mentioned DL model.

2. Materials and methods

2.1. Mice and tumor system

Pathogen-free inbred adult male mice of BALB/c (H-2^d) strain were used at 8–12 weeks of age. The mice were procured from the animal house facility of the Banaras Hindu University approved by the institutional animal ethical committee and kept in the animal rooms of the School of Biotechnology. Mice received food and water *ad libitum* and were treated with utmost humane care. For all purposes, mice were sacrificed by cervical dislocation. DL is maintained in ascitic form by serial transplantation in BALB/c mice or in an *in vitro* cell culture system by serial passage. Irrespective of whether the DL cells were obtained from the *in vitro* culture system maintained as suspension cultures or from the ascitic fluid, they exhibited similar phenotypic features.

2.2. Reagents

All reagents used were of tissue culture or analytical grade. Tissue culture medium RPMI 1640 was purchased from Hyclone (USA), supplemented with 20 µg/ml gentamycin, 100 µg/ml streptomycin, 100 IU penicillin purchased from Himedia (India) and 10% fetal calf serum from Hyclone (USA), henceforth, referred to as complete medium. Sodium Dichloroacetate (>99% pure containing up to <1% Sodium Bicarbonate) was obtained from BuyDCA.com (Sonora CA). DCFDA was purchased from Sigma–Aldrich (USA). Antibodies against Bcl2, β-actin, CAD, IFN-γ, IL-10, MCT1, VATPase, (Imgenex, USA), Caspase-3 (Sigma–Aldrich, USA), HSP70, IL-6, p53 (Chemicon International, UK), GLUT1 (Affinity Bio Reagents, USA), were purchased from the indicated sources. Secondary antibodies conjugated to alkaline phosphatase were obtained from Bangalore Genie (India). Primers for RT-PCR were purchased from Abion International AG (Germany) and Integrated DNA Technologies (USA). BCIP/NBT was purchased from Amresco (USA). TUNEL assay

kit was purchased from Invitrogen (USA). Annexin-V assay kit was purchased from Imgenex (USA).

2.3. Cell cultures

Single cell suspension of thymocytes was prepared following previously described standard procedures [24] from freshly isolated thymus of mice. Colo205 cell line (colon adenocarcinoma) was obtained from National Centre For cell Science (NCCS), Pune (India) and other cell lines U87 (glioblastoma), AGS (gastric adenocarcinoma), SiHa (cervical cancer) and MCF7 (breast adenocarcinoma) were gifted by Dr. A.C. Bharti, Institute of Cytology and Preventive Oncology (ICPO), Noida (India). Thymocytes, DL (Tumor) cells and cell lines namely: Colo205, U87, AGS, SiHa & MCF7 cells (1×10^6 cells/ml) were incubated in 96 well culture plate in complete RPMI medium with or without the indicated concentrations of DCA at 37 °C in humidified atmosphere of a CO₂ (5% CO₂) incubator. DCA was solubilized in PBS (pH 7.3 ± 0.1) at a concentration of 8.0 M for preparation of a stock solution and further diluted in complete medium before adding to cell cultures at the indicated final concentration.

2.4. Cell survival assay

Survival of cells was estimated by a standard MTT assay according to a method described earlier [18] with slight modifications. MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] was dissolved in PBS at a concentration of 5 mg/ml. MTT solution (50 µl) was added to each well of the culture plate containing tumor cells in 200 µl medium and incubated at 37 °C for 4 h. The medium was then carefully removed, without disturbing the dark blue formazan crystals. Fifty microliters of DMSO was added to each well and mixed thoroughly to dissolve the formazan crystals. Plates were then read on a microplate reader (Labsystems, Finland) at a wavelength of 540 nm. Data is presented as % survival, which is calculated by normalizing the O.D. values of experimental groups against control.

2.5. Analyses of apoptosis

Induction of apoptosis in tumor cells was estimated by multiple methods as described earlier [18], including enumeration of apoptotic cell population by Wright–Giemsa, TUNEL & Annexin-V along with estimation of % DNA fragmentation.

2.5.1. Wright–Giemsa staining

Cell suspensions were smeared on a slide and air-dried, fixed in methanol, stained with Wright–Giemsa staining solution, mounted on glycerine, and analyzed under light microscope (Carl Zeiss, Germany) at 400× magnification. Apoptotic cells were identified on the basis of morphological features that included contracted cell bodies; condensed, uniformly circumscribed and densely stained chromatin; and membrane bound apoptotic bodies containing one or more nuclear fragments. The percentage of apoptotic cells was determined by counting more than 300 cells in at least 3 separate microscopic fields.

2.5.2. TUNEL staining

Apoptotic cells were identified by TUNEL staining using a TUNEL assay kit (Invitrogen, USA), following to the manufacturer's instructions. Briefly, DL cells were fixed in 1% paraformaldehyde solution in PBS at 4 °C for 15 min followed by incubation in 70% ethanol at –20 °C for 30 min. Cells were then incubated in DNA labeling solution containing TdT enzyme and BrdUTP at 37 °C for 60 min followed by washing with rinse buffer and incubation in Alexa Fluor 488 dye-labeled anti-BrdU antibody for 30 min at room

temperature. Apoptotic cells were identified both under phase contrast and fluorescence optics. Cells which fluoresced brightly were apoptotic when observed under fluorescence optics of fluorescence microscope (Nikon, Japan).

2.5.3. Annexin-V staining for detection of apoptotic cells

DL cells were stained with annexin-V-FITC apoptosis detection kit (Imgenex, USA). DL cells were washed thrice with PBS and resuspended in binding buffer containing annexin-V-FITC reagent. After incubation at room temperature for 20 min, the cells were observed under fluorescence microscope (Nikon, Japan). Cells showing dark green fluorescence were determined as apoptotic and also simultaneously confirmed for apoptotic morphology under phase contrast optics.

2.5.4. Estimation of percent DNA fragmentation

Treated or untreated DL cells were lysed in 0.5 ml of Tris-EDTA buffer (pH 7.4) containing 0.2% (v/v) Triton X-100 and the fragmented DNA was separated from intact chromatin in a microfuge tube (labeled as B) by centrifugation at 13,000g at 4 °C for 10 min. Supernatant containing the fragmented DNA was transferred to another microfuge tube (labeled as T). A volume of 0.5 ml of 25% TCA was added to each T and B tubes and vortexed vigorously. DNA was precipitated overnight at 4 °C and collected at 13,000g at 4 °C for 10 min. Supernatant was discarded and 80 µl of 5% TCA was added to each pellet. DNA was hydrolyzed by heating at 90 °C for 15 min. At this stage, a blank was included containing 80 µl of 5% TCA. Then 160 µl of freshly prepared diphenylamine (DPA) reagent (150 mg diphenylamine in 10 ml glacial acetic acid, 150 µl concentrated H₂SO₄ and 50 µl of acetaldehyde solution) was added and the tubes were allowed to stand overnight at room temperature to develop color. One hundred microlitre of this colored solution was transferred to the wells of a 96-well flat-bottomed ELISA plate and absorbance was measured at 600 nm in a microtitre ELISA plate reader (Labsystems, Finland). Percent DNA fragmentation was calculated as:

$$\text{DNA fragmentation (\%)} = [T / (T + B)] \times 100$$

where *T* = absorbance of fragmented DNA and *T* + *B* = absorbance of total DNA.

2.6. ELISA for detection of cytokines

A standard ELISA was performed to detect the presence of indicated cytokines following a method described earlier [17]. Briefly, polystyrene microwell plates (Tarsons, Kolkata, India) were coated with 10 µg of protein sample and incubated overnight at 4 °C. In the negative control, test samples were not added to wells of ELISA plates and were processed for subsequent steps in the same ways as described for the experimental sets. The plates were washed with 0.15 M PBS containing 0.1% (v/v) Tween 20 (PBS-Tween). Unbound sites were saturated with PBS containing 1% bovine serum albumin (BSA). The plates were again washed with PBS-Tween followed by addition of antibodies against the indicated proteins at a dilution of 1:1000. The plates were incubated at 37 °C for 60 min followed by addition of 50 µl of *p*-nitrophenyl phosphate (NPP) (1 mg/ml) in enzyme substrate buffer. The absorbance was measured after 10 min at 405 nm in an ELISA plate reader (Labsystems, Finland) and the cytokines level is presented as pg/ml.

2.7. Western immunoblot analysis

Western immunoblot analysis for detection of indicated proteins was carried out following a method described earlier [18]. Cells were washed with chilled PBS and lysed in 50 µl of lysis buffer (20 mM Tris-Cl, pH 8.0, 137 mM NaCl, 10% (v/v) glycerol, 1% (v/v)

v) Triton X-100, 2 mM EDTA; 1 mM phenylmethylsulfonyl fluoride, 20 µM leupeptin containing aprotinin at 0.15 U ml⁻¹) for 20 min at 4 °C. Protein content in each sample was determined by using standard Bradford method. Twenty microgram of Triton X-100 solubilized proteins was separated on 10% SDS-polyacrylamide gel at 20 mA. The gel was processed further for immunoblotting. The separated proteins were transferred onto a nitrocellulose membrane (Sartorius, Germany) (1.5 h at 150 mA), immunoblotted with antibodies against HSP70, p53, Bcl2, Caspase-3, CAD, GLUT1, MCT1, and VATPase and probed with a secondary antibody: anti-rabbit IgG conjugated to alkaline phosphatase and detected by a BCIP/NBT solution (Amresco, USA). Equal loading of proteins was determined by using equal cell number for preparation of lysates, loading of equal protein content and immunoblotting of β-actin.

2.8. RT-PCR for expression of mRNA of PUMA, HIF1-α, GLUT1 and MCT1

RT-PCR analysis for the expression of mRNA for indicated genes and β-actin were carried out according to a method described earlier [16] using a one step RT-PCR cell to cDNA kit (Ambion, USA). Primer sequences for various genes are shown in Table 1. PCR was performed for 15 min to make cDNA at 50 °C. The amplification was carried out for 30 cycles with initial denaturation at 94 °C for 2 min followed by annealing (annealing temperature as per respective primer design) for 30 s and elongation at 72 °C for 30 s. The samples were separated on an agarose gel (1%) containing ethidium bromide (0.3 µg/ml). Bands were visualized and analyzed on a UV-transilluminator (Biorad, Australia).

2.9. Measurement of intracellular reactive oxygen species (ROS)

ROS measurement was carried out as described earlier by Furu-ta et al. (2008) [25] with slight modifications. Untreated or tumor cells treated with DCA for 3 h were washed followed by incubation with HBSS containing the fluorescent dye dichlorodihydrofluorescein diacetate, (DCFDA) at a final concentration 0.1 mM. The cells were further incubated at 37 °C for 45 min, followed by washing with PBS. The cells stained with dye were visualized under fluorescence microscope (Nikon, Japan) at a magnification of 400× and photographed. The amount of staining was quantified by MCID software.

2.10. Estimation of lactate

Lactate concentration in culture supernatant was measured using an enzymatic colorimetric method (Spinreact, Granada, Spain) as described by Somoza et al. (2007) [26]. Briefly, 1 µl sample was diluted in 200 µl 50 mM PIPES (pH 7.5) containing 4-chlorophenol (4 mM), lactate oxidase (800 U/l), peroxidase (2000 U/l), and 4-aminophenazone (0.4 mM), followed by incubation for

Table 1
Primer sequences for RT-PCR analysis.

Genes	Primer sequences
HIF-1α	F-5'-CTCAAAGTCGGACAGCCTCA-3' R-5'-CCCTGCAGTAGGTTCTGCT-3'
PUMA	F-5'-CAGACTGTGAATCCTGTCT-3' R-5'-ACAGTATCTTACAGGCTGGG-3'
GLUT1	F-5'-CTTTGTGGCCTTCTTGAAG-3' R-5'-CCACACAGTTGCTCCACAT-3'
MCT1	F-5'-CCATTGTGGAATGCTGCTCT-3' R-5'-CCTACTTCTTCCCCATCC-3'
β-Actin	F-5'-GGCACAGTGTGGGTGAC-3' R-5'-CTGGCACCACCTTCTAC-3'

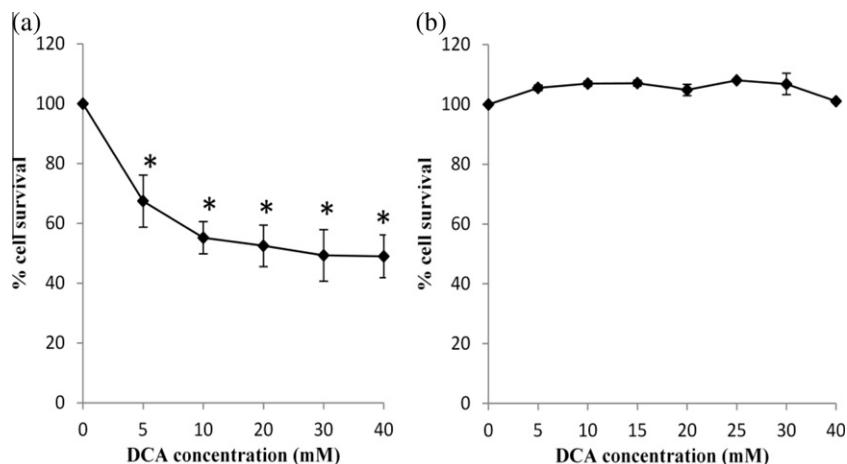


Fig. 1. Effect of DCA on the survival of tumor cells and thymocytes *in vitro*. Tumor cells (1×10^6 cells/ml) were incubated for 24 h in medium alone or containing indicated concentrations of DCA (a) followed by estimation of tumor cell survival by MTT assay as described in materials and methods. Thymocytes (1×10^6 cells/ml) were incubated in medium alone or containing the indicated concentrations of DCA (b) followed by estimation of cell survival by MTT assay as described in materials and methods. Values shown are mean \pm SD of three independent experiments done in triplicate. * $p < 0.05$ vs. values of tumor cells incubated in medium alone.

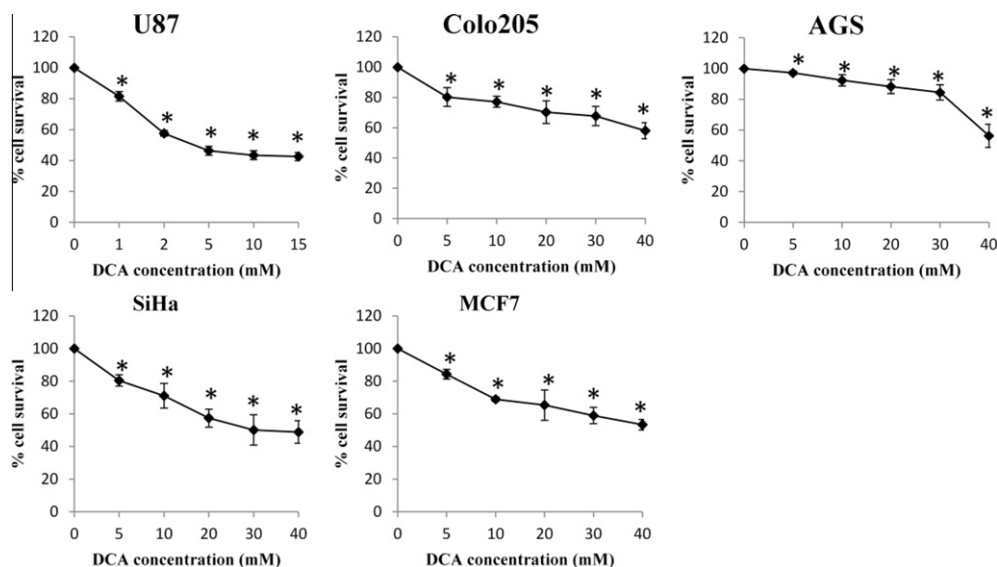


Fig. 2. Antineoplastic effect of DCA on cell lines of different origins. Cells (1×10^6 cells/ml) were incubated for 24 h with the mentioned doses of DCA followed by estimation of cell survival by MTT assay as described in materials and methods. Values shown are mean \pm SD of three independent experiments done in triplicate. * $p < 0.05$ vs. values of cells incubated in medium alone.

10 min at room temperature, and measurement of absorbance at 505 nm was recorded. Lactate concentration was expressed in milligram per deciliter.

2.11. Quantification of glucose

Glucose content in culture supernatant was measured using a commercial kit from Agappe diagnostics LTD, Kerala, India based on conversion of glucose to H_2O_2 by converting it into a colored red quinine product by the action of peroxidase as described by Chaudhuri et al. (2006) [27]. Briefly, 10 μ l of culture supernatant sample was mixed with 1 ml of working reagent containing phosphate buffer (pH 7.4), phenol, glucose-oxidase, peroxidase and 4-aminoantipyrine and was incubated for 10 min at 37 $^{\circ}$ C. The final reading was taken at 505 nm. Glucose concentration was expressed in milligram per deciliter.

2.12. Statistical analysis

All experiments were conducted thrice in triplicate. The statistical significance of differences between test groups was analyzed using SIGMASTAT[®] software to calculate 1-way analysis of variance (ANOVA) with student Neuman-Kuels post hoc comparisons. The difference was considered significant when p was less than 0.05.

3. Results

3.1. DCA reduces the survival of tumor cells but not thymocytes *in vitro*

Tumor cells (1×10^6 cells/ml) were incubated in medium alone or containing the indicated concentrations of DCA for 24 h followed by estimation of cell survival by MTT assay. Results are

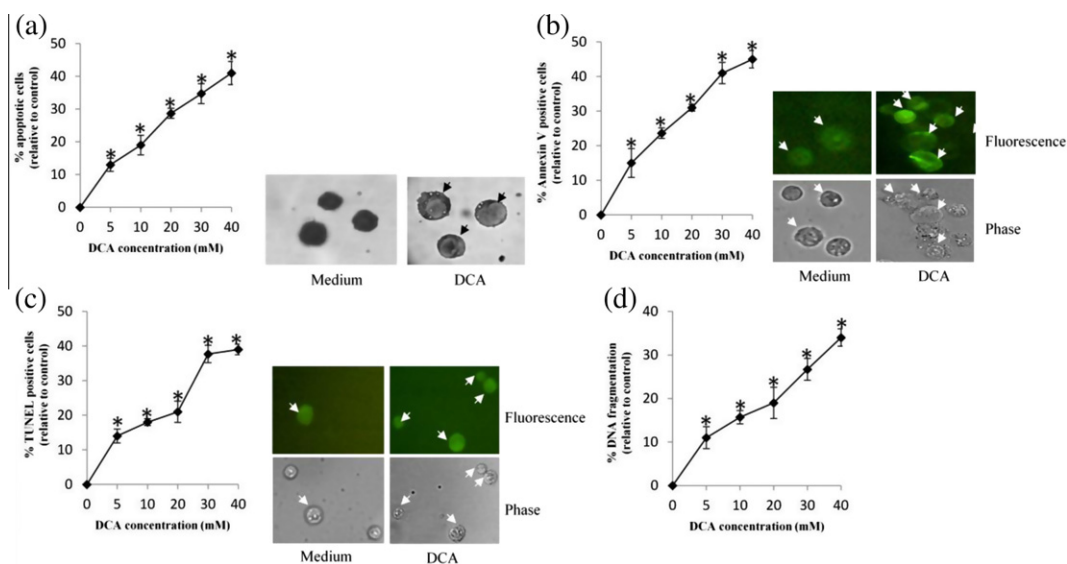


Fig. 3. Effect of DCA on the induction of apoptosis in tumor cells. Tumor cells (1×10^6 cells/ml) were incubated for 24 h in medium alone or containing the indicated concentrations of DCA followed by enumeration of apoptotic cell population by Wright-Giemsa (a), Annexin-V (b) and TUNEL staining (c) and estimation of DNA fragmentation (d) as described in materials and methods. The percentage of apoptotic cells was determined by counting more than 300 cells in at least 3 separate microscopic fields. Plates shown in each right panel are from a representative experiment in which tumor cells were incubated in the presence or absence of DCA (30 mM) for 24 h to show the phenotype of the apoptotic cells analyzed by each respective method. Arrows indicate the apoptotic cells. Values shown are mean \pm SD of three independent experiments done in triplicate. * $p < 0.05$ vs. values for DL cells incubated in medium alone. Plates shown (a,b,c) are from a representative experiment out of three independent experiments with similar results.

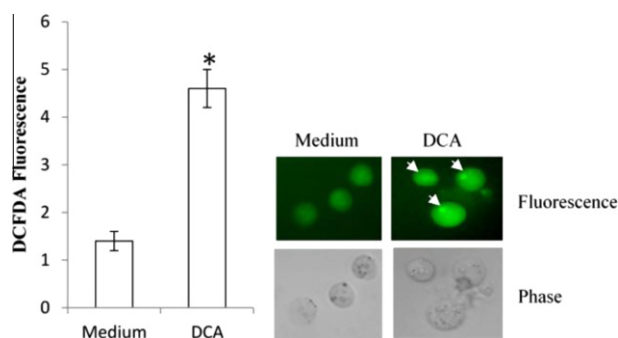


Fig. 4. Effect of DCA on the production of ROS in tumor cells. Tumor cells (1×10^6 cells/ml) were incubated in medium alone or DCA (30 mM) containing medium for 3 h followed by estimation of ROS production as described in materials and methods. Values shown are mean \pm SD of three independent experiments done in triplicate. * $p < 0.05$ vs values for DL cells incubated in medium alone. Plates shown are from a representative experiment out of three independent experiments with similar results. Arrows indicate DCFDA positive cells.

shown in Fig. 1(a). Incubation of tumor cells in medium containing DCA resulted in a significant dose-dependent decline in tumor cell survival compared to tumor cells incubated in medium alone. We observed the optimum inhibitory effect of DCA on tumor cell survival at 30 mM concentration. Thymocytes (1×10^6 cells/ml) were incubated in medium containing indicated concentrations of DCA or medium alone for 24 h followed by estimation of cell survival by MTT assay. Results are shown in Fig. 1(b). DCA treatment of thymocytes did not alter their survival, indicating that DCA at the concentrations checked, was non-cytotoxic for normal cells.

3.2. DCA manifests antineoplastic action against cell lines of varying etiology

Indicated cell lines (1×10^6 cells/ml) were incubated in medium alone or containing the mentioned dose range of DCA for

24 h prior to estimation of cell survival by MTT assay. Results are shown in Fig. 2. DCA treatment resulted in a dose dependent significant inhibition of cell survival compared to respective untreated controls.

3.3. DCA induces apoptosis in tumor cells

Tumor cells (1×10^6 cells/ml) were incubated in medium with or without the indicated concentration of DCA for 24 h and induction of apoptosis was confirmed by Wright-Giemsa (Fig. 3a), Annexin-V (Fig. 3b), TUNEL (Fig. 3c) staining and estimation of percent DNA fragmentation (Fig. 3d). DCA was found to induce apoptosis in DL cells in a dose dependent manner.

3.4. Effect of DCA treatment on the production of ROS in tumor cells

As ROS production is reported to be one of the earliest response events following DCA treatment [10], tumor cells (1×10^6 cells/ml) were incubated in medium with or without DCA (30 mM) for 3 h followed by determination of ROS production as described in materials and methods. Results are shown in Fig. 4. The production of ROS in DCA-treated tumor cells was found to be significantly increased compared to control.

3.5. Modulation of cell survival regulatory genes and proteins in DCA-treated tumor cells

Tumor cells (1×10^6 cells/ml) were incubated in medium alone or containing DCA (30 mM) for 24 h, followed by RT-PCR and immunodetection for the expression of HIF1- α and PUMA genes and HSP70, Bcl2, p53, caspase-3 and CAD proteins. Results are shown in Fig. 5. DCA treatment of tumor cells resulted in an inhibition in the expression of HIF1- α gene along with HSP70 and Bcl2 proteins whereas the expression of PUMA at the mRNA level (Fig. 5a) and p53, caspase-3 and CAD at the protein level (Fig. 5b) was found to be augmented in comparison to the respective controls in both mRNA and protein levels.

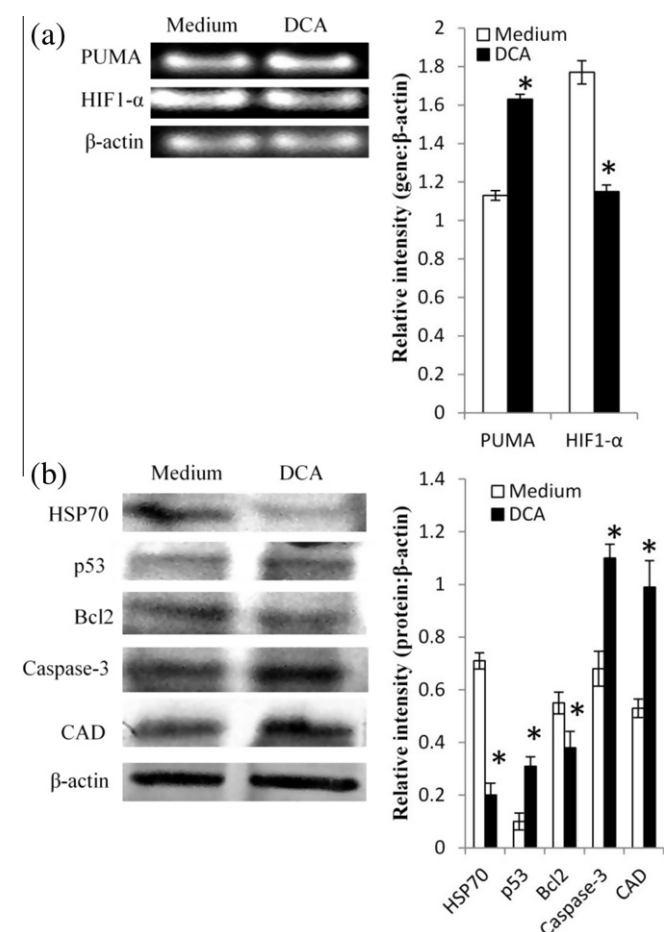


Fig. 5. Effect of DCA on the expression of cell survival regulatory genes and proteins in tumor cells. Tumor cells (1×10^6 cells/ml) were treated for 24 h *in vitro* in medium alone or containing DCA (30 mM) followed by analysis of the expression of cell survival regulatory genes HIF-1 α and PUMA (a) by RT-PCR and proteins HSP70, Bcl2, p53, caspase-3 and CAD (b) by immunoblotting as described in materials and methods. Bands shown are from a representative experiment out of three independent experiments done in triplicate. Values shown in bar diagrams are mean \pm SD of densitometric analysis of the bands. Densitometric analysis was carried out by comparing the density of indicated proteins/genes against the β -actin by using a 'Quantity One' software of Biorad. Y axis shows relative intensity.

3.6. Effect of DCA treatment on the expression GLUT1 and pH regulators in tumor cells

Tumor cells (1×10^6 cells/ml) were incubated in medium with or without DCA (30 mM) for 24 h followed by RT-PCR of GLUT1 and MCT1 and immunodetection of GLUT1, MCT1 and VATPase by western blotting. Results are shown in Fig. 6. The expression of GLUT1, MCT1 and VATPase was found to be inhibited following DCA treatment of tumor cells compared to respective controls.

3.7. Reversal in biophysical composition of conditioned medium of DCA-treated tumor cells *in vitro*

Tumor cells (1×10^6 cells/ml) were incubated in medium alone or containing DCA (30 mM) for 24 h followed by measurement of pH and estimation of lactate and glucose in culture supernatant. Results are shown in Table 2. A significant increase in the level of pH and glucose was observed in the culture supernatant of DCA-treated tumor cells compared to control. The level of lactate was

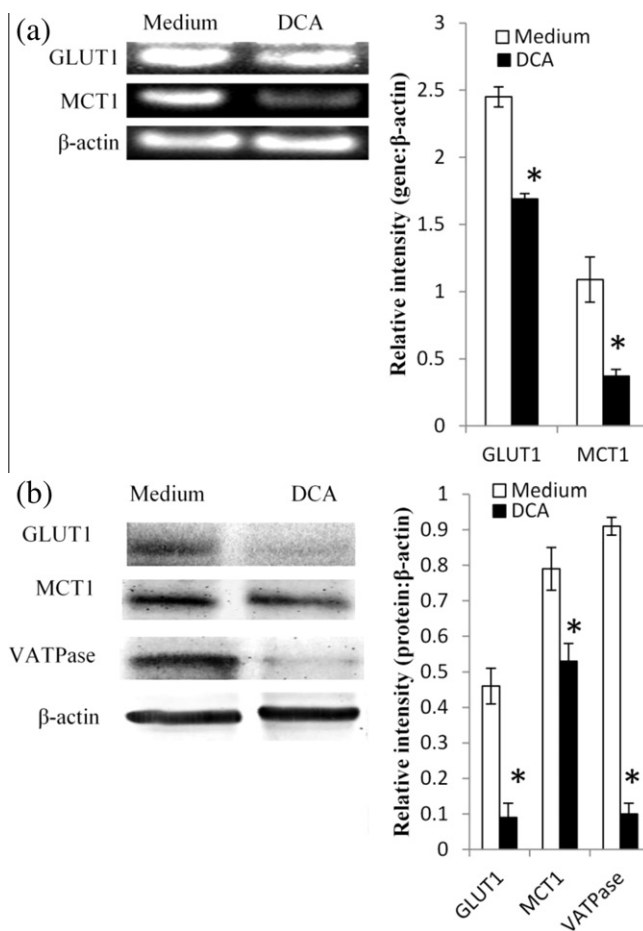


Fig. 6. DCA treatment inhibits the expression of GLUT1 and pH regulatory genes and proteins in tumor cells. Tumor cells (1×10^6 cells/ml) were incubated *in vitro* for 24 h in medium alone or containing DCA (30 mM) followed by processing of cells for RT-PCR and immunoblot analysis of the indicated genes (a) and proteins (b). Bands shown are from a representative experiment out of three independent experiments done in triplicate. Values shown in bar diagrams are mean \pm SD of densitometric analysis of the bands. Densitometric analysis was carried out by comparing the density of indicated proteins/genes against the β -actin by using a 'Quantity One' software of Biorad. Y axis shows relative intensity.

Table 2

Modulation in the biophysical composition of conditioned medium of DCA-treated tumor cells.

Parameters	In vitro treatment	
	Medium	DCA
Glucose (mg/dl)	63.3 \pm 0.26	107.1 \pm 0.30*
Lactate (mg/dl)	57.7 \pm 0.35	40.3 \pm 0.28*
pH	7.1 \pm 0.02	7.5 \pm 0.03*

Tumor cells (1×10^6 cells/ml) were incubated for 24 h in medium alone or containing DCA (30 mM) followed by estimation of glucose, lactate and pH in cell-free culture supernatant as described in materials and methods. Values shown are mean \pm SD of three independent experiments done in triplicates.

* $p < 0.05$ vs. values for tumor cells incubated in medium alone.

found to significantly decline in the culture supernatant of tumor cells treated with DCA compared to control.

3.8. Modulation in the production of cytokines in DCA-treated tumor cells

Cell-free culture supernatants obtained from tumor cells (1×10^6 cells/ml) incubated in medium alone or containing DCA

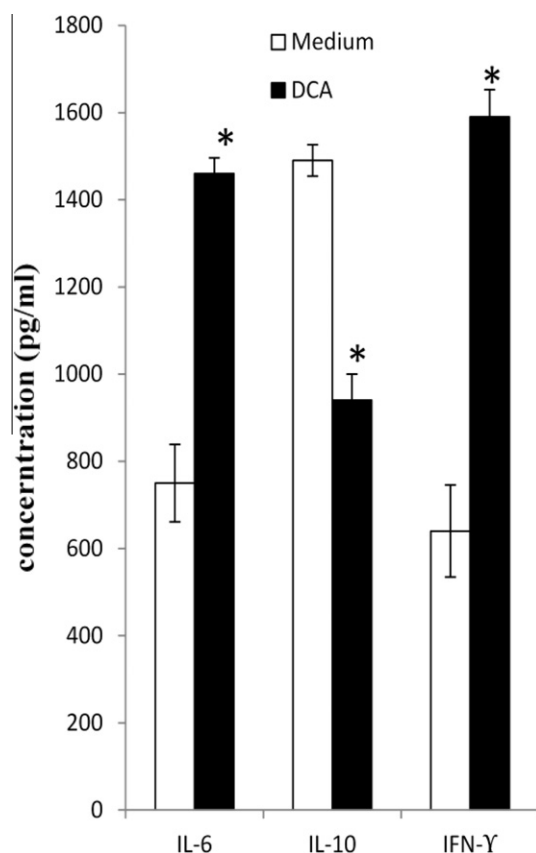


Fig. 7. *In vitro* treatment of tumor cells with DCA modulates cytokines production. Cell-free culture supernatant obtained from tumor cells incubated in medium alone or containing DCA (30 mM) were immunodetected for the indicated cytokines by ELISA as described in material and methods. Values shown in are mean \pm SD of three independent experiments done in triplicate. * $p < 0.05$ vs. values for tumor cells incubated in medium alone.

(30 mM) for 24 h, were immunodetected for the presence of the indicated cytokines by ELISA. Results are shown in Fig. 7. We observed a significant increase in the level of IFN- γ and IL-6 along with a decline of IL-10 in the culture supernatant of DCA-treated tumor cells compared to untreated control.

4. Discussion

In the present investigation, we demonstrate the antitumor action of DCA against tumor cells of hematological origin, moreover, DCA also shows antineoplastic action against cell lines of various etiology indicating its broad spectrum of antitumor potential. The inhibitory action of DCA on cell survival was DL specific as DCA showed no effect on survival of thymocytes. Moreover, the inhibitory effect of DCA on tumor cell survival was associated with an augmented induction of apoptosis. Thus we next explored the molecular mechanisms of DCA-induced inhibition of tumor cell survival and apoptosis. Reactive oxygen species (ROS) have been shown to play an indispensable role in DCA-induced apoptosis in a variety of malignant cells [4,5,8,10]. DCA augments the generation of ROS by allowing entry of NADH produced by TCA cycle to complex I of the electron transport chain associated with reactivation of PDH activity [4,5]. Similarly, in our case also we found a significantly augmented ROS production in DCA-treated tumor cells, which may constitute one of the mechanisms of DCA-induced tumor cell death. Moreover, we also observed augmented expression of p53, PUMA, caspase-3 and CAD along with a decline

of HIF1- α and Bcl2. It has been reported that ROS oxidizes and thus activates p53 [28], which in turn promotes apoptosis by regulating the expression level of two main cell survival regulatory molecules viz. PUMA and HIF1- α , respectively [29,30]. Further, the altered expression of PUMA and HIF1- α is demonstrated to down-regulate the expression of anti-apoptotic protein Bcl2 [31,32], which in turn promotes caspase-3 and CAD activation along with declined expression of HIF1- α [33,34], culminating in induction of apoptosis. Nevertheless, DCA also induces apoptosis in tumor cells by decreasing mitochondrial membrane hyperpolarization resulting in activation of pro-apoptotic mediators [4,5,35,36]. Therefore, the aforementioned mechanisms could also underlie DCA-induced augmented apoptosis observed in our study. Interestingly, we also observed a declined expression of HSP70 protein in DCA-treated tumor cells, which has been demonstrated to play a central role in regulating cell survival [37,38]. To the best of our knowledge, this is the first report showing the inhibition of HSP70 expression in tumor cells by DCA. The declined expression of HSP70 has been correlated to a decline in the expression of HIF1- α [39,40]. Moreover, it has been found that the expression of Bcl2 and activation of caspases regulated by HSP70 [41–43].

We also found an elevation in the level of glucose in tumor-conditioned medium concomitant to a declined expression of GLUT1 in DCA-treated tumor cells. Review of literature reveals that GLUT1 regulates the uptake of glucose inside in tumor cells [44–46]. Thus the over-expression of GLUT1 can be correlated with tumor progression and its poor prognosis [44–47]. Moreover, GLUT1 expression is modulated by HIF1- α , HSP70 and p53 [48–50]. Therefore, the inhibited GLUT1 expression observed in DCA-treated tumor cells could be attributed to a declined HIF1- α and HSP70 along with an increased expression of p53. We also observed an increase in the pH of conditioned medium of DCA-treated tumor cells, which could be correlated to a low level of lactate along with an inhibited expression of two key pH regulators: MCT1 and VAPase, of which MCT1 is a transporter of lactate [51]. The low level of lactate in tumor-conditioned medium can also be associated to a declined glucose uptake by DCA-treated tumor cells owing to: (1) a down-regulated GLUT1 expression; and (2) shifting of tumor cell metabolism from glycolysis to glucose oxidation. Nevertheless, extracellular pH of tumor cells has been reported to play a very crucial role in tumor cell survival by modulating expression of HIF1- α and its downstream genes [52,53]. Therefore, the increased extra-cellular pH of DCA-treated tumor cells as observed in our study may also be correlated to the declined expression of HIF1- α . The possible targeted genes downstream to HIF1- α could be GLUT1 and HSP70 the expression of which was observed to get modulated in DCA-treated tumor cells. This report shows for the first time the effect of DCA on expression of pH regulators and GLUT1 in tumor cells by which it may contribute to the modulation in the composition of extracellular milieu of tumor cells, triggering induction of apoptosis.

Another novel observation of the present investigation demonstrates modulated production of cytokines by DCA-treated tumor cells. DCA treatment of tumor cells resulted in a down-regulated expression of IL-10, whereas IL-6 and IFN- γ were up-regulated. The altered cytokine balance may inhibit tumor cell survival by modulating expression of cell survival regulatory molecules like Bcl2, p53, and GLUT1 [54–57] along with pH regulators: like MCT1 and VAPase [18,19]. As summarized in Fig. 8 DCA triggers tumor cell death by ushering multiple molecular mechanisms culminating in induction of apoptosis. In conclusion, this study presents the novel molecular mechanisms underlying DCA-induced apoptosis in T cell lymphoma and thus may provide a new insight for antineoplastic therapeutic application of DCA.

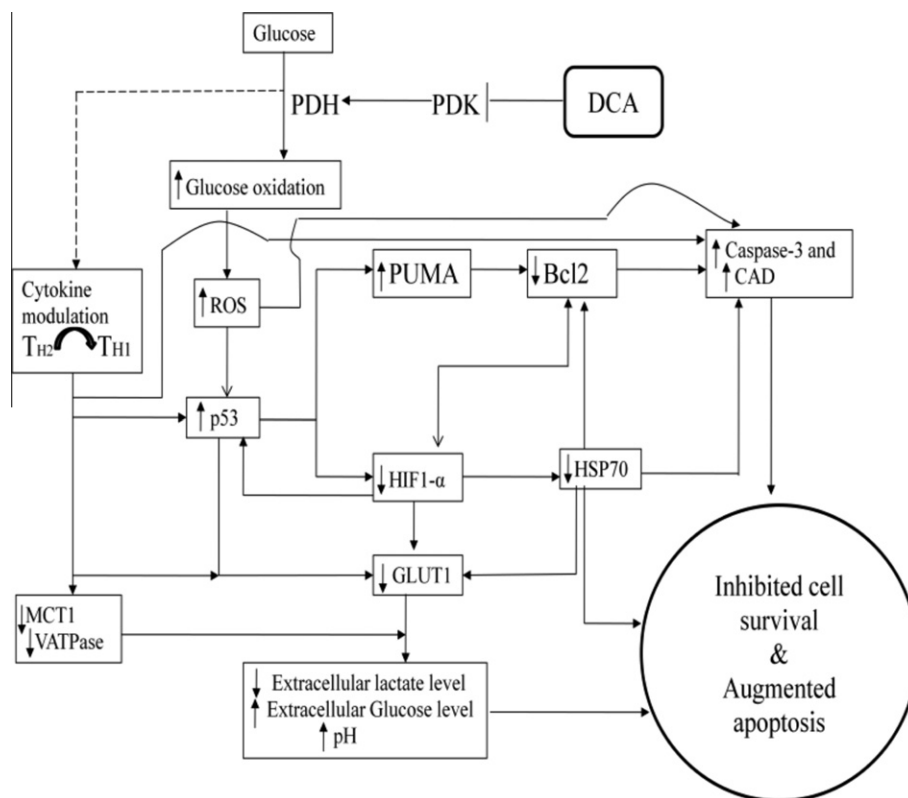


Fig. 8. Summary of molecular mechanisms implicated in DCA-induced tumor cell apoptosis. Summary indicates the multiple pathways and molecules involved in inhibition of tumor cell survival by DCA.

Acknowledgments

The financial support to the School of Biotechnology from DBT and DST PURSE program, Government of India, New Delhi is acknowledged. The authors express gratitude to CSIR and DBT, New Delhi, for fellowship support to Ajay Kumar (09/013(0329)/2010-EMR-I) and Shiva Kant (DBT-JRF/2010-11/79), respectively. Cell lines were kind gift from Dr. Alok Chandra Bharti, Institute of Cytology and Preventive Oncology (ICPO), Noida, India is thankfully acknowledged. The help of Dr. S.D. Singh of Parul Pathology laboratory is gratefully acknowledged for some biochemical assays.

References

- [1] R.A. Gatenby, R.J. Gillies, Why do cancers have high aerobic glycolysis?, *Nat Rev. Cancer* 4 (2004) 891–899.
- [2] R.J. Gillies, R.A. Gatenby, Adaptive landscapes and emergent phenotypes: why do cancers have high glycolysis?, *J Bioenerg. Biomembr.* 39 (2007) 251–257.
- [3] E.D. Michelakis, L. Webster, J.R. Mackey, Dichloroacetate (DCA) as a potential metabolic-targeting therapy for cancer, *Br. J. Cancer* 99 (2008) 989–994.
- [4] E.D. Michelakis, G. Sutendra, P. Dromparis, L. Webster, A. Haromy, E. Niven, C. Maguire, T.L. Gammer, J.R. Mackey, D. Fulton, B. Abdulkarim, M.S. McMurtry, K.C. Petruk, Metabolic modulation of glioblastoma with dichloroacetate, *Sci. Transl. Med.* 2 (2010) 31–34.
- [5] 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.
- [6] 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.
- [7] B.M. Madhok, S. Yeluri, S.L. Perry, T.A. Hughes, D.G. Jayne, Dichloroacetate induces apoptosis and cell-cycle arrest in colorectal cancer cells, *Br. J. Cancer* 102 (2010) 1746–1752.
- [8] R.C. Sun, P.G. Board, A.C. Blackburn, Targeting metabolism with arsenic trioxide and dichloroacetate in breast cancer cells, *Mol. Cancer* 10 (2011) 142.
- [9] Y. Wong, G.S. Huggins, M. Debidia, N.C. Munshi, I. De Vivo, Dichloroacetate induces apoptosis in endometrial cancer cells, *Gynecol. Oncol.* 109 (2008) 394–402.
- [10] L.H. Stockwin, S.X. Yu, S. Borgel, C. Hancock, T.L. Wolfe, L.R. Phillips, M.G. Hollingshead, D.L. Newton, Sodium dichloroacetate selectively targets cells with defects in the mitochondrial ETC, *Int. J. Cancer* 127 (2010) 2509–2510.
- [11] S. Nakamura, World Health organization (WHO) classification of malignant lymphoma-how is the WHO now?, *Gan to Kagaku ryoho* 31 (2004) 149–157.
- [12] A. Moll, A. Niwald, M. Gratek, M. Stolarska, Ocular complications in leukemia and malignant lymphoma in children, *Klin Oczna* 106 (2004) 783–787.
- [13] J. Zhou, K. Maurer, L. Farina, J.G. Gribben, The role of the tumor microenvironment in hematological malignancies and implication for therapy, *Front. Biosci.* 10 (2005) 1581–1596.
- [14] A.C. Bharti, S.M. Singh, Inhibition of macrophage nitric oxide production by gangliosides derived from a spontaneous T cell lymphoma: the involved mechanisms, *Nitric Oxide* 8 (2003) 75–82.
- [15] M.P. Singh, H. Sharma, S.M. Singh, Prolactin promotes growth of a spontaneous T cell lymphoma: role of tumor and host derived cytokines, *Cancer Invest.* 24 (2006) 601–610.
- [16] V. Gupta, S.M. Singh, Gender dimorphism of tumor growth: role of gonadal hormones in differential regulation of apoptosis of a murine T cell lymphoma, *J. Biomed. Sci.* 15 (2008) 147–162.
- [17] V. Singh, S.M. Singh, A tumour stage-dependent evolution of drug resistant T cell lymphoma: role of soluble mediators of tumour and host origin, *Leuk. Res.* 33 (2009) 700–709.
- [18] N.K. Vishvakarma, S.M. Singh, Mechanisms of tumor growth retardation by modulation of pH regulation in the tumor-microenvironment of a murine T cell lymphoma, *Biomed. Pharmacother.* 65 (2011) 27–39.
- [19] A. Kumar, N.K. Vishvakarma, A. Tyagi, A.C. Bharti, S.M. Singh, Anti-neoplastic action of aspirin against a T-cell lymphoma involves an alteration in the tumour microenvironment and regulation of tumour cell survival, *Biosci. Rep.* 32 (2012) 91–104.
- [20] G. Klein, Comparative studies of mouse tumors with respect to their capacity for growth as 'Ascitic tumors' and their average nucleic acid content, *Exp. Cell res.* 2 (1951) 518–524.
- [21] H. Goldie, M.D. Felix, Growth characteristics of free tumor cells transformed serially in the peritoneal fluid of mouse, *Cancer Res.* 11 (1951) 73–80.
- [22] M. Udaychander, A. Menakshi, R. Muthiah, R. Sivanandham, Tumor targeting of liposomes encapsulating Ga-67 and antibody to Dalton's lymphoma associated antigen (anti-DLAA), *Int. J. Radiat. Oncol., Biol., Phys.* 13 (1987) 1713–1718.

- [23] D. Khynriam, S.B. Prasad, Cisplatin induced genotoxic effects and endogenous glutathione levels in mice bearing as cites Dalton's lymphoma, *Mutat. Res.* 526 (2003) 9–18.
- [24] A. Shanker, S.M. Singh, Characterization of factors inducing apoptosis in thymocytes of mice bearing a transplantable T-cell lymphoma of spontaneous origin, *Neoplasma* 47 (2000) 90–95.
- [25] E. Furuta, S.K. Pai, R. Zhan, S. Bandyopadhyay, M. Watabe, Y.Y. Mo, S. Hirota, S. Hosobe, T. Tsukada, K. Miura, S. Kamada, K. Saito, M. Iizumi, W. Liu, J. Ericsson, K. Watabe, Fatty acid synthase gene is up-regulated by hypoxia via activation of Akt and sterol regulatory element binding protein-1, *Cancer Res.* 68 (2008) 1003–1011.
- [26] B. Somoza, R. Guzman, V. Cano, B. Merino, P. Ramos, C. Diez-Fernandez, Induction of cardiac uncoupling protein-2 expression and adenosine 5'-monophosphate- activated protein kinase phosphorylation during early states of diet-induced obesity in mice, *Endocrinology* 148 (2007) 924–931.
- [27] R.K. Chaudhuri, M. Mukherjee, D. Sengupta, S. Mazumder, Limitation of glucose oxidase method of glucose estimation in jaundiced neonates, *Indian J. Exp. Biol.* 44 (2006) 254–255.
- [28] B. Liu, Y. Chen, D.K. St Clair, ROS and p53: a versatile partnership, *Free Radic. Biol. Med.* 44 (2008) 1529–1535.
- [29] J.R. Jeffers, E. Parganas, Y. Lee, C. Yang, J. Wang, J. Brennan, K.H. MacLean, J. Han, T. Chittenden, J.N. Ihle, P.J. McKinnon, J.L. Cleveland, G.P. Zambetti, Puma is an essential mediator of p53-dependent and -independent apoptotic pathways, *Cancer Cell* 4 (2003) 321–328.
- [30] D. Chen, M. Li, J. Luo, W. Gu, Direct interactions between HIF-1 alpha and Mdm2 modulate p53 function, *J. Biol. Chem.* 278 (2003) 13595–13598.
- [31] M.T. Hemann, S.W. Lowe, The p53-Bcl-2 connection, *Cell Death Differ.* 13 (2006) 1256–1259.
- [32] J.T. Erler, C.J. Cawthorne, K.J. Williams, M. Koritzinsky, B.G. Wouters, C. Wilson, C. Miller, C. Demonacos, I.J. Stratford, C. Dive, Hypoxia-mediated down-regulation of Bid and Bax in tumors occurs via hypoxia-inducible factor 1-dependent and -independent mechanisms and contributes to drug resistance, *Mol. Cell Biol.* 24 (2004) 2875–2889.
- [33] D. Trisicuglio, C. Gabellini, M. Desideri, E. Ziparo, G. Zupi, D. Del Bufalo, Bcl-2 regulates HIF-1alpha protein stabilization in hypoxic melanoma cells via the molecular chaperone HSP90, *PLoS One* 5 (2010) 11772.
- [34] A. Iervolino, D. Trisicuglio, D. Ribatti, A. Candiloro, A. Biroccio, G. Zupi, D. Del Bufalo, Bcl-2 overexpression in human melanoma cells increases angiogenesis through VEGF mRNA stabilization and HIF-1-mediated transcriptional activity, *FASEB J.* 16 (2002) 1453–1455.
- [35] S. Vella, M. Conti, R. Tasso, R. Cancedda, A. Pagano, Dichloroacetate inhibits neuroblastoma growth by specifically acting against malignant undifferentiated cells, *Int. J. Cancer* 130 (2012) 1484–1493.
- [36] W. Cao, S. Yacoub, K.T. Shiverick, K. Namiki, Y. Sakai, S. Porvasnik, C. Urbanek, 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.
- [37] V. Dudeja, N. Mujumdar, P. Phillips, R. Chugh, D. Borja-Cacho, R.K. Dawra, S.M. Vickers, A.K. Saluja, Heat shock protein 70 inhibits apoptosis in cancer cells through simultaneous and independent mechanisms, *Gastroenterology* 136 (2009) 1772–1782.
- [38] M. Rohde, M. Daugaard, M.H. Jensen, K. Helin, J. Nylandsted, M. Jäättelä, Members of the heat-shock protein 70 family promote cancer cell growth by distinct mechanisms, *Genes Dev.* 19 (2005) 570–582.
- [39] W. Luo, J. Zhong, R. Chang, H. Hu, A. Pandey, G.L. Semenza, Hsp70 and CHIP selectively mediate ubiquitination and degradation of hypoxia-inducible factor (HIF)-1alpha but not HIF-2alpha, *J. Biol. Chem.* 285 (2010) 3651–3663.
- [40] D. Zhang, J. Li, M. Costa, J. Gao, C. Huang, JNK1 mediates degradation HIF-1alpha by a VHL-independent mechanism that involves the chaperones Hsp90/Hsp70, *Cancer Res.* 70 (2010) 813–823.
- [41] B. Jiang, P. Liang, G. Deng, Z. Tu, M. Liu, X. Xiao, Increased stability of Bcl-2 in HSP70-mediated protection against apoptosis induced by oxidative stress, *Cell Stress Chaperones* 16 (2011) 143–152.
- [42] J. Kaur, J. Kaur, R. Ralhan, Induction of apoptosis by abrogation of HSP70 expression in human oral cancer cells, *Int. J. Cancer* 85 (2000) 1–5.
- [43] E.Y. Komarova, E.A. Afanasyeva, M.M. Bulatova, M.E. Cheetham, B.A. Margulis, I.V. Guzova, Downstream caspases are novel targets for the antiapoptotic activity of the molecular chaperone hsp70, *Cell Stress Chaperones* 9 (2004) 265–275.
- [44] V. Ganapathy, M. Thangaraju, P.D. Prasad, Nutrient transporters in cancer: relevance to Warburg hypothesis and beyond, *Pharmacol. Ther.* 121 (2009) 29–40.
- [45] M.L. Macheda, S. Rogers, J.D. Best, Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer, *J. Cell. Physiol.* 202 (2005) 654–662.
- [46] N.C. Denko, Hypoxia, HIF1 and glucose metabolism in the solid tumour, *Nat. Rev. Cancer* 8 (2008) 705–713.
- [47] C.D. Young, A.S. Lewis, M.C. Rudolph, M.D. Ruehle, M.R. Jackman, U.J. Yun, O. Ilkun, R. Pereira, E.D. Abel, S.M. Anderson, Modulation of glucose transporter 1 (GLUT1) expression levels alters mouse mammary tumor cell growth *in vitro* and *in vivo*, *PLoS One* 6 (2011) 23205.
- [48] P. Vaupel, The role of hypoxia-induced factors in tumor progression, *Oncologist* 9 (2004) 10–17.
- [49] A. Ikari, M. Nakano, K. Kawano, Y. Suketa, Up-regulation of sodium-dependent glucose transporter by interaction with heat shock protein 70, *J. Biol. Chem.* 277 (2002) 33338–33343.
- [50] F. Schwartzberg-Bar-Yoseph, M. Armoni, E. Karnieli, The tumor suppressor p53 down-regulates glucose transporters GLUT1 and GLUT4 gene expression, *Cancer Res.* 64 (2004) 2627–2633.
- [51] S. Bröer, Lactate transportation is required for lymphocyte activation, *Nat. Chem. Biol.* 1 (2005) 356–357.
- [52] C. Willam, C. Warnecke, J.C. Schefold, J. Kügler, P. Koehne, U. Frei, M. Wiesener, K.U. Eckardt, Inconsistent effects of acidosis on HIF-alpha protein and its target genes, *Pflugers Arch.* 451 (2006) 534–543.
- [53] B.A. Webb, M. Chimenti, M.P. Jacobson, D.L. Barber, Dysregulated pH: a perfect storm for cancer progression, *Nat. Rev. Cancer* 11 (2011) 671–677.
- [54] L. Wall, F. Burke, C. Barton, J. Smyth, F. Balkwill, IFN-gamma induces apoptosis in ovarian cancer cells in vivo and in vitro, *Clin. Cancer Res.* 9 (2003) 2487–2496.
- [55] Y.H. Park, S.K. Sohn, J.G. Kim, M.H. Lee, H.S. Song, M.K. Kim, J.S. Jung, J.J. Lee, H.J. Kim, D.H. Kim, Interaction between BCL2 and interleukin-10 gene polymorphisms alter outcomes of diffuse large B-cell lymphoma following rituximab plus CHOP chemotherapy, *Clin. Cancer Res.* 15 (2009) 2107–2115.
- [56] P. Klausen, L. Pedersen, J. Jurlander, H. Baumann, Oncostatin M and interleukin 6 inhibit cell cycle progression by prevention of p27kip1 degradation in HepG2 cells, *Oncogene* 19 (2000) 3675–3683.
- [57] A.R. Shikhman, D.C. Brinson, J. Valbracht, M.K. Lotz, Cytokine regulation of facilitated glucose transport in human articular chondrocytes, *J. Immunol.* 167 (2001) 7001–7008.