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Full Length Research Paper

In vitro and *in vivo* antitumoral activitiy of sodium dichloroacetate (DCA-Na) against murine melanoma

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Normal cells typically use mitochondrial oxidative phosphorylation to metabolize glucose and switch over to glycolysis only in hypoxic or anoxic environment. However, cancer cells avidly consume glucose for energy by glycolysis to survive in the hypoxic environment of malignant lesions, a phenomenon known as the Warburg effect, the glycolytic phenotype offers growth advantages to cancer cells by resisting apoptosis. This unique metabolic characteristic of cancer cells has identified the mitochondrion as a target for cancer therapy. *In vitro*, different cancer cells were treated with DCA-Na, all cancer cell lines were affected by the treatment with DCA-Na, the B16F10 cell line was the most sensitive to the treatment, decreased the cellular viability, induced cellular death by apoptosis, affecting different metabolic pathways (LDH, PKA/cAMP, HIF-1α, and GAPDH), decreased the capacity of cellular invasion and angiogenesis. *In vivo* the DCA-Na treatment induced 20% survival and decreased the tumoral diameter, volume and weight, without affect the body weight and avoid metastasis in C57BL/6 mice. Our results suggest that DCA-Na is a good candidate as a new agent in the treatment of cancer due to its antitumoral properties *in vitro* and *in vivo*.

Key words: Cancer, sodium dichloroacetate, glycolysis, melanoma.

INTRODUCTION

Conversion of glucose to lactic acid in the presence of oxygen is known as aerobic glycolysis or the 'Warburg effect'. Increased aerobic glycolysis is uniquely observed in cancers [corroborated by FdG PET (18-fluorodeoxyglucose positron emission tomography) studies in metastatic human cancers]. Because early carcinogenesis occurs in a hypoxic microenvironment, the transformed cells initially have to rely on glycolysis for energy production (Gatenby and Gillies, 2004). However, this early metabolic adaptation appears to also offer a proliferative advantage, suppressing apoptosis. Furthermore, the ''byproducts'' of glycolysis (that is, lactate and acidosis) contribute to the breakdown of the extracellular matrix, facilitate cell mobility, and increase the metastatic potential (Plas and Thompson, 2002). At the seems the metabolic phenotype in cancer is due to a potentially plasticity mitochondrial remodeling that results in suppressed oxidative phosphorylation, enhanced glycolysis, and suppressed apoptosis (Stacpoole et al., 1998). The growth of various tumors has been linked to the cAMP/PKA pathway because this complex may activate different proteins involved in signal transduction and cellular growth; in lung cancer cells, the increase in PKA type I isozyme induces a nontumorigenic phenotype, while its decrease is followed by acquisition

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Abbreviations: DCA-Na, Sodium dichloroacetate; **LDH,** lactate dehydrogenase; **PKA/cAMP,** cAMP-dependent protein kinase A; **TUNEL,** terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; **HIF-1α,** hypoxia-inducible factor 1-alpha.

of tumorigenic properties and the deregulation of the effector molecule, cAMP, is involved in cancer genesis and the reduction in cAMP has an anti-proliferative effect on colorectal cancer cells. Several data reveal that the cAMP/PKA signaling pathway is altered in different cancers, and may be exploited for cancer diagnosis and/or therapy (Caretta and Mucignat, 2011). LDH-A belonging to the isoforms of LDH, is expressed and involved in the growth and tumor maintenance of breast cancer cells. LDH catalyzes the conversion of pyruvate to lactate of the cellular glycolytic process (Fantin et al., 2006). The ability of DCA to decrease lactate production has been used for more than 30 years in the treatment of lactic acidosis that complicates inherited mitochondrial diseases in humans (Stacpoole et al., 1998). DCA inhibits the activity of pyruvate dehydrogenase kinase (PDK), thereby stimulating the mitochondrial enzyme pyruvate dehydrogenase (PDH). When turned off, PDH no longer converts pyruvate to acetyl-CoA required for mitochondrial respiration and glucose dependent oxidative phosphorylation. DCA thus shifts cellular metabolism from glycolysis to glucose oxidation, decreasing the mitochondrial membrane potential gradient and helping to open mitochondrial transition pores. This metabolic switch facilitates translocation of pro-apoptotic mediators like cytochrome *c* (cyt c) and apoptosis inducing factor (AIF), both of which stimulate apoptosis. DCA thereby drives cancer cells to commit suicide by apoptosis (Shanta and Lippard, 2009). DCA is currently in phase III clinical trials for the treatment of chronic lactic acidosis in congenital mitochondrial disorders and 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 (Sun et al., 2010). The aim of this study is to evaluate the effect of DCA-Na on viability and glycolytic metabolism of B16F10 melanoma cancer cells *"In vitro"* and tumor growth and survival in a model of murine melanoma "*In vivo"*.

MATERIALS AND METHODS

Main reagents

Penicillin-streptomycin solution, ficoll-hypaque solution, trypsin-EDTA solution, RPMI-1640 medium, Dulbecco's modified Eagle's medium (DMEM/F-12), and 1% antibiotic-antimycotic solution were obtained from (Life Technologies GIBCO, Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Sigma-Aldrich (St. Louis, MO).

DCA-Na

The DCA-Na (150.92451 M.W.) was purchased from HYMERA LAB (Monterrey, N.L., México). It was filtered (0.2 µm of diameter, Millipore, USA) and diluted to a stock of 37.5 M in DMEM/F-12 culture medium.

Cell culture

B16F10, MCF-7, INER-51, MDA-MB 231, Huh-7, Hela, TC-1, PC3 and DU145 cancer cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in DMEM/F-12 supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution. Cells were grown to confluence at 37°C, and 5% CO_2 atmosphere.

Macrophages harvest and culture

Resident peritoneal cells from 6-8 weeks old female C57BL/6 mice (obtained from Harlan, México) were harvested and cultured as described by Gómez et al. (1997). Murine peritoneal macrophages were seeded into 96-well plates (Costar, Cambridge, MA) at 5×10^3 cells/well in DMEM-F12 medium and treated with DCA-Na at concentrations of 0-750 mM/ml and incubated at 37°C in 5% CO_2 atmosphere for 72 h. Thereafter, we determined the effect of DCA-Na on the macrophages viability by MTT method.

Cell viability

Cancer cell lines (5×10^3cells/well) were plated on 96 flat-bottom well plates, and incubated 24 h at 37°C in 5% CO_2 atmosphere. After incubation, culture medium was removed, and DCA-Na diluted in the same medium was added at concentrations ranging from 0-750 mM/ml. The plates were then incubated for 24, 48, and 72 h at 37°C, and 5% CO_2 atmosphere. Thereafter, the supernatant was removed and cells were washed twice with DMEM/F-12 medium. Cell viability was determined by the MTT method, and cytotoxicity was expressed as the concentration of 20% (LD_{20}), 50% (LD_{50}), and 90% (LD_{90}) cell growth inhibition. Results were given as the mean ±SD of three independent experiments.

Detection of treatment-induced apoptosis

To assess the degree of treatment-induced apoptosis and/or necrosis in B16F10 cells, the cells were exposed to DCA-Na and cultured as described above. Apoptosis was determined by acridine orange/ethidium bromide nuclear stain, and confirmed by the terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay.

Acridine orange/ethidium bromide

Briefly, B16F10 cancer cells were seeded at 1×10^6 cells/well into 6-well plates in DMEM/F12 + 5% FBS and incubated overnight. The DCA-Na at concentrations of 20% (LD_{20}), 50% (LD_{50}) and 90% (LD_{90}) cell growth inhibition, was added to the cells and incubated for 48 h. After one washing with phosphate-buffered saline (PBS), the cells were stained with 2 µL of a mixture (1:1) of acridine orange-ethidium bromide (100 µg/ml) in PBS. The cells were incubated for five minutes in the dark at room temperature and washed with phosphate-buffered saline; then were viewed under a Nikon inverted fluorescent microscope (TE-Eclipse 300) with an attached camera and photographs were taken under fluorescent conditions. Detection of apoptosis was based on morphological and fluorescent characteristics of the stained cells. Viable cells were indicated by bright green color, apoptotic cells by orange/brown color, and necrotic cells by red color**.** Each experiment was done in triplicate and repeated at least three times.

Tunel

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) was performed with TACS 2 TdT-DAB *In Situ* Apoptosis Detection kit (Trevigen, Gaithersburg, Maryland, USA), following the manufacturer's instructions. Briefly, after culture B16F10 cells at 5×10^3 cells/well and treated with DCA-Na LD_{50} and LD_{90}, by 48 h. The cells were digested with proteinase K at a concentration of 20 µg/ml for 15 min. Endogenous peroxidase activity was quenched with 2% H_2O_2 for 5 min. The cells were immersed in terminal deoxynucleotidyl transferase (TdT) buffer. TdT, 1 mM Mn^{2+}, and biotinylated dNTP in TdT buffer were then added to cover the cells and incubated in a humid atmosphere at 37°C for 60 min. The cells were washed with PBS and incubated with streptavidin-horseradish peroxidase for 10 min. After rinsing with PBS, the cells were immersed in DAB solution. The cells were counterstained for 3 min with 1% methyl green. Cells containing fragmented nuclear chromatin characteristic of apoptosis will exhibit brown nuclear staining that may be very dark after labeling.

Senescence-associated β-galactosidase

B16F10 cells at 1×10^6 cells/well in six well plates and treated with doxorubicin (0.005 µg/ml) as a positive control, DCA-Na LD_{50} and LD_{90}, by 48 h. Thereafter, washing in PBS and fixation for 3 to 5 min (room temperature) in 2% formaldehyde/0.2% glutaraldehyde, the cells were incubated overnight at 37°C (without CO_2) with freshly prepared senescence associated β-galactosidase (SA-β-gal) (BioVision, Mountain View, CA) stain solution (1 mg/ml X-gal, 40 mM citric acid/sodium phosphate pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$). Cells were then rinsed with PBS, and 100 to 200 cells in six microscopic fields were counted to determine the percentages of SA-β-gal stained positive cells.

LDH assay

The LDH is an enzyme widely distributed in mammalian tissues which in the presence of NAD/NADH, converts lactate to pyruvate (Andre et al., 2004).. LDH activity was measured by Cytotoxicity Detection Kit (LDH) (Roche Applied Science, IN, USA), is based on the release of the cytosolic enzyme, LDH, from cells with damaged cellular membranes. Thus, in cell culture, the course of drug-induced cytotoxicity can be followed quantitatively by measuring the activity of LDH in the supernatant (Delgado et al., 2004). The B16F10 cancer cells (5×10^3 cells/well) were treated with different doses of DCA-Na in a range of 0 to 750 mM/ml for 24 and 48 h. Thereafter, they were washed twice with ice-cold PBS, harvested by centrifugation at 250 g for 10 min at 25°C, and the supernatants were used for the activity assay according to the manufacturer's instructions. Optical densities resulting from LDH activity were measured in a microplate reader at 490 nm. Results were given as the mean ±SD of three independent experiments.

Determination of the cAMP dependent protein kinase (PKC) activity

The B16F10 cells (5×10^6 cells/T25 flasks) were treated with LD_{20}, LD_{50}, and LD_{90} of DCA-Na and cultured by 48 h. Thereafter, the detection of protein kinase C or cAMP Dependent protein kinase was determined by a commercial kit (Pep Tag for Non-Radioactive Detection of protein kinase C or cAMP Dependent protein kinase Promega, Madison, USA) according to the manufacturer's instructions. The cells were harvested and crude cell extracts were prepared and a volume of crude extract containing 60 µg protein was used to measure PKA activity with PepTag A1 peptide as substrate for PKA, and the phosphorylation reaction was incubated at room temperature for 10 min. The cAMP-dependent protein kinase catalytic subunit provided by the kit was used as positive control. The phosphorylation reaction without cAMP-dependent protein kinase catalytic subunit and without protein extract served as negative control. Total PKA activity in the presence of exogenously added 5 mM cAMP was determined. This experiment was conducted three times with similar results. Fluorescence intensity of bands on the 0.8% agarose gel was measured by densitometry with Quantity One Basic 2006 software (BIO RAD USA). Protein concentrations were determined by Bio-Rad protein assay (Bio-Rad Laboratories, CA. USA).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA from 1×10^6 B16F10 cancer cells was prepared using 1 ml of TRIzol (Gibco, Life Technologies, Grand Island, NY, USA). The concentration and integrity of the RNA was determined by measuring absorbance at 260 nm and analyzed by electrophoresis on 1% agarose gels. For RT-PCR, 5 µg of the total RNA was reverse transcribed using SUPERSCRIPT™ II RT (Gibco, Laboratories, Life Technologies, NY, USA) and oligo $(dT)_{12-18}$ primer (Gibco, Laboratories Life Technologies, NY, USA). The cDNA (1.25 µL) obtained was amplified using Taq DNA polymerase (Gibco, Laboratories, Life Technologies, NY, USA), and specific primers for β-actin Forward $^{5'}$GAC TTC GAG CAA GAG AT, Reverse $^{3'}$ TCG TCA TAC GCC TGC TTG CT$^{5'}$, GAPDH Forward $^{5'}$ACC ACA GTC CAT GCC ATC AC, Reverse $^{3'}$ TCC ACC ACC CTG TTG CTG TA$^{5'}$, and 18S ribosomal Forward $^{5'}$ GCC CCC TCG ATG CTC TTA G, Reverse $^{3'}$ AAA TGC TTT CGC TCT GGT CC$^{5'}$. The amplified DNA sizes were 430, 452, and 251 bp, respectively. The sizes and amounts of PCR products were analyzed by electrophoresis in 1.2% agarose gels, visualized by ethidium bromide staining with UV light, photographed and OD quantified using a scanning densitometer (UVP Labworks Media Cybernetics Gel-Pro Analyzer™ Software, MA, USA).

DNA binding-activity of HIF-1α

1×10^7 B16F10 cells were plated in T-75 flasks and allowed to attach overnight. Hypoxia was achieved or not by the incubation with or without the presence of $CoCl_2$ (50 mM) (Sigma Chemical Company, St Louis, MI, USA) during 4 h (Huang et al., 2003). Thereafter, the cells were treated with LD_{50}, or LD_{90} of DCA-Na after 48 h of incubation at 37°C in 5% CO_2 atmosphere, cell-free supernatants were isolated following centrifugation at 600 g for 10 min and stored in sterile tubes (Eppendorf, USA) at -20°C until analysis. HIF-1α was measured in the supernatants using a commercial ELISA kit (HIF-1α Transcription Factor Assay Cayman Chemical kit, USA) according to the manufacturer's instructions. The reactions were terminated by the addition of 100 µL of stop solution. Absorbance readings (450 nm) obtained from individual supernatant samples were converted to amounts of HIF-1α DNA binding using a standard curve produced with dilutions of recombinant mouse HIF-1α for each assay.

Test of cellular invasion

The 6-well Matrigel Invasion Chamber (BD BioCoat™ Matrigel ™ BD Biosciences, Bedford, MA) was prepared according to instructions of manufacturer. For induced chemoattraction 2.5 ml of serum fetal bovine at 5% was added to each well and immediately covered with the Cell Culture Inserts containing an 8 µ pore size PET membrane with a thin layer of MATRIGEL Basement

Membrane Matrix. The layer occludes the pores of the membrane, blocking non-invasive cells from migrating through the membrane. In contrast, invasive cells (malignant and non-malignant) are able to detach themselves from and invade through the Matrigel Matrix and the 8 µ membrane pores. Thereafter, 1×10^6 B16F10 cells/well were added and treated with LD_{50} or LD_{90} of DCA-Na and incubated at 37°C in 5% CO_2 atmosphere for 22 and 48 h. After the non-invading cells were removed from the upper surface of the membrane by scrubbing (Cell scraper, Corning Incorporated Costar, México) and the cells were stained and fixed by hematoxylin and eosin method. The invading cells were observed under microscope at 40-200X magnifications, counted cells in several fields of duplicate membranes and the data is expressed as the percent invasion through the Matrigel Matrix and membrane relative to the migration through the control membrane according to the next formula.

$$\% \text{ Invasion} = \frac{\text{Mean number of cells invading through Matrigel insert membrane}}{\text{Mean number of cells migrating through control insert membrane}} \times 100$$

Angiogenesis determination

Forty microlitre of cell-based extracellular matrix gel per well was added in 96-well tissue culture plates and incubated for 1 h at 37°C to solidify. Thereafter, 5×10^3 B16F10 cells/well were plated and allowed to attach overnight. The cells were treated with LD_{50} or LD_{90} of DCA-Na, or PMA 1 µM (angiogenesis stimulation) or JNJ 1 µM (angiogenesis inhibition) and incubated at 37°C in 5% CO_2 atmosphere for 24 and 48 h. Thereafter, 10 µL of fluorescent dye Calcein AM solution (1:100) per well was added for visualization of cellular organization by fluorescence microscopy (Nikon inverted fluorescent microscope TE-Eclipse 300).

Effects of DCA-Na on the mice inoculated with B16F10 cells

To determine the effects of DCA-Na on the tumoral growth and survival in mice inoculated with B16F10 cells; female C57BL/6 mice, 8 weeks old, were obtained from the Harlan, México, S. A. de C. V. The mice were maintained on pelleted food and water *ad libitum*. B16F10, a melanoma cell line of C57BL/6 origin, was cultured and trypsinized, and the harvested cells were washed twice and resuspended in saline solution at 5×10^5 cellular concentration. Ten mice per group were injected subcutaneously with 5×10^5 B16F10 cells in the right flank, shaved previously, and treated intraperitoneally with DCA-Na twice at day at doses of 500 and 1000 mg/kg or 0.2 ml saline solution; at the seven days after the cellular inoculation. The mice were monitored until death or recovery. Tumor volume was recorded every 7 days using a measuring gauge (PRETUL, USA) using the formula: volume=length × $(\text{width})^2$. In parallel, another group received similar treatments, but were sacrificed by cervical dislocation at 28 days before dying from tumors growth; the tumors were surgically collected, and tumor weight and metastasis were determined (metastasis was determined during the necropsy by findings of tumor cells in muscle, peritoneal cavity, bowel, and liver).

Statistical analysis

All experiments were performed in triplicate, and statistical analysis was performed using analysis of variance (ANOVA) followed by Least Significant Difference (LSD). The results were considered statistically significant if the *p value was <0.05.

RESULTS

In vitro cellular cytotoxicity assays

The ability of DCA-Na to promote cell death was evaluated by the MTT assay using B16F10, MCF-7, INER-51, MDA-MB 231, Huh-7, Hela, TC-1, PC3 and DU145 cancer cell lines; as well as murine peritoneal macrophages. Results are presented in Figures 1, 2, and 3. When evaluated the effect of treatment at different doses by 72 h, the relative cell viability of murine peritoneal macrophages was not affected (Figure 4), but the cytotoxic effect was observed on all the cancer cell lines treated (Figures 1 and 2) in a dose dependent manner (*$p<0.05$). We choose the B16F10 cell line for the next studies because it was the most sensitive to the treatment, and the LD_{20} (75 mM/ml), LD_{50} (300 mM/ml), and LD_{90} (750 mM/ml) were determined at the 24, 48, and 72 h of treatment, shown a decreased of the cellular viability in a dose and time dependent manner (Figure 3).

DCA-Na induces apoptosis in B16F10 cancer cells

Characteristic morphological changes were assessed at doses of LD_{20} (75 mM/ml), LD_{50} (300 mM/ml), and LD_{90} (750 mM/ml) of DCA-Na treatment at 48 h by fluorescent microscopy using acridine orange and ethidium bromide staining. The results showed a significant amount of apoptotic cell death induced by the treatment in the B16F10 cells when compared with the control cells (Figure 5). The TUNEL assay corroborated this result and, not apoptotic cells were observed among the control B16F10 cells, whereas abundant apoptosis characterized by complex of brown color in the interior of the cells, was observed with the treatment of DCA-Na at doses of LD_{50} (300 mM/ml) and LD_{90} (750 mM/ml) (Figure 6).

Senescence cell

A well established marker of senescence is the expression of SA-β-gal in cells. The positive control of senescence is induced by doxorubicin (*$p<0.05$). The DCA-Na treatment LD_{50} (300 mM/ml) and LD_{90} (750 mM/ml) did not induce senescence in B16F10 cells (*$p<0.05$) similar to the control B16F10 cells (Figure 7).

Evaluation of glycolytic activity

The GAPDH is an enzyme involved in the glycolysis

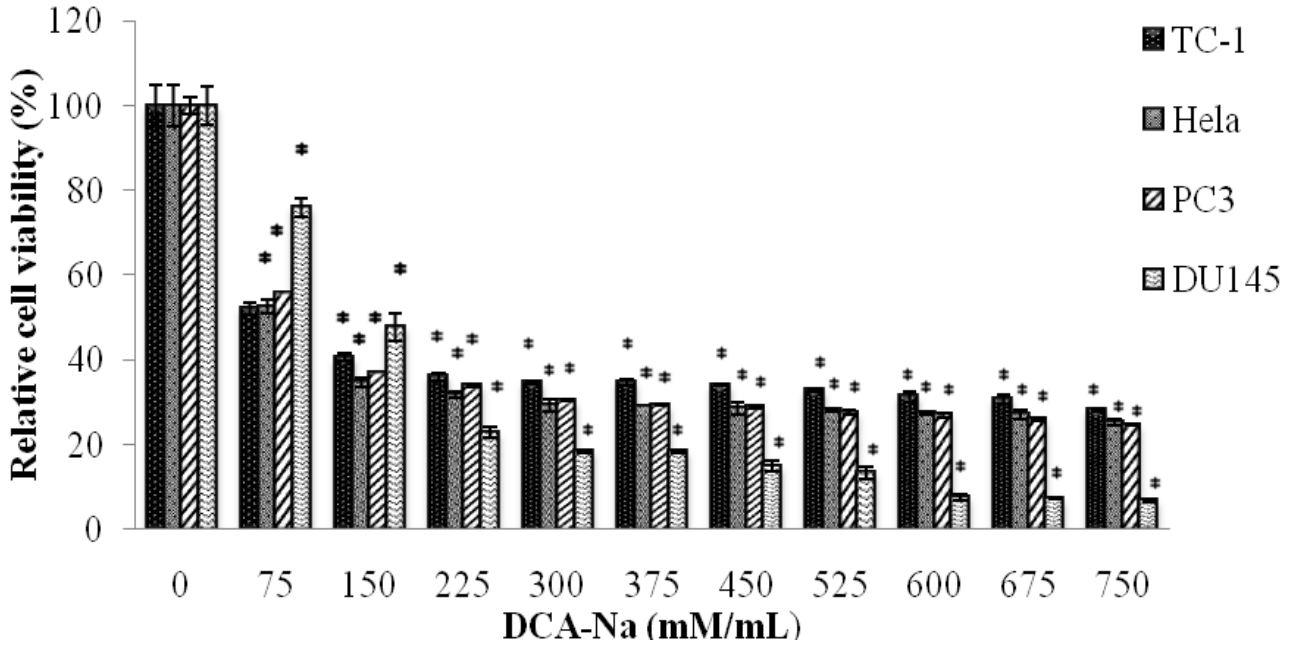

Figure 1. Cell viability of TC-1, Hela, PC3, and DU145 cell lines treated with DCA-Na. TC-1, Hela, PC3, and DU145 cells (5×10^3cells/well) were cultured into 96 well plates and incubated overnight. Thereafter, the plates were treated with DCA-Na concentrations ranging from 0 to 750 mM/ml, and incubated for 72 h at 37°C, and 5% CO_2 atmosphere. Thereafter, a standard MTT assay was performed. The optical density was determined at 570 nm. Data represent means of triplicate samples with ±SD indicated. *p<0.05 as compared with untreated cells.

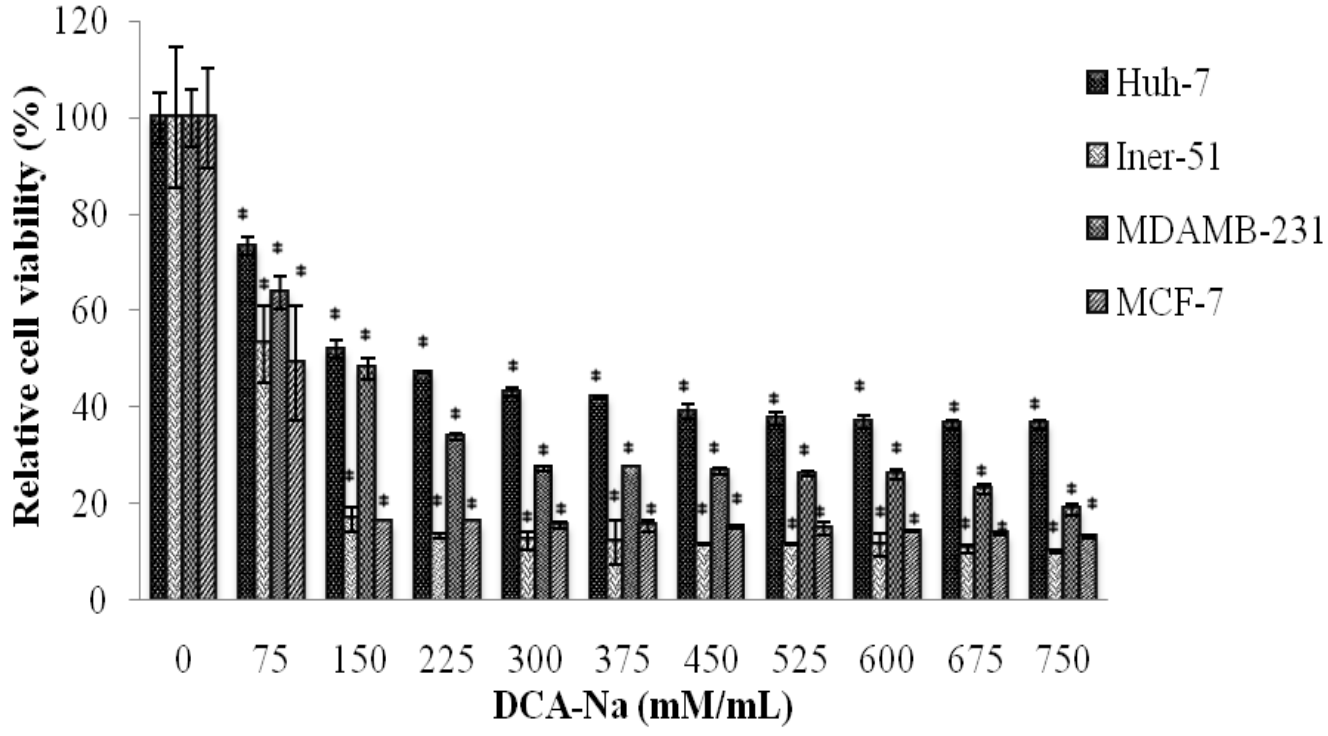

Figure 2. Cell viability of Huh-7, Iner-51, MDAMB-231, and MCF-7 cell lines treated with DCA-Na. Huh-7, Iner-51, MDAMB-231, and MCF-7 cells (5×10^3 cells/well) were cultured into 96 well plates and incubated overnight. Thereafter, the plates were treated with DCA-Na concentrations ranging from 0 to 750 mM/ml, and incubated for 72 h at 37°C, and 5% CO_2 atmosphere. Thereafter, a standard MTT assay was performed. The optical density was determined at 570 nm. Data represent means of triplicate samples with ±SD indicated. *p<0.05 as compared with untreated cells.

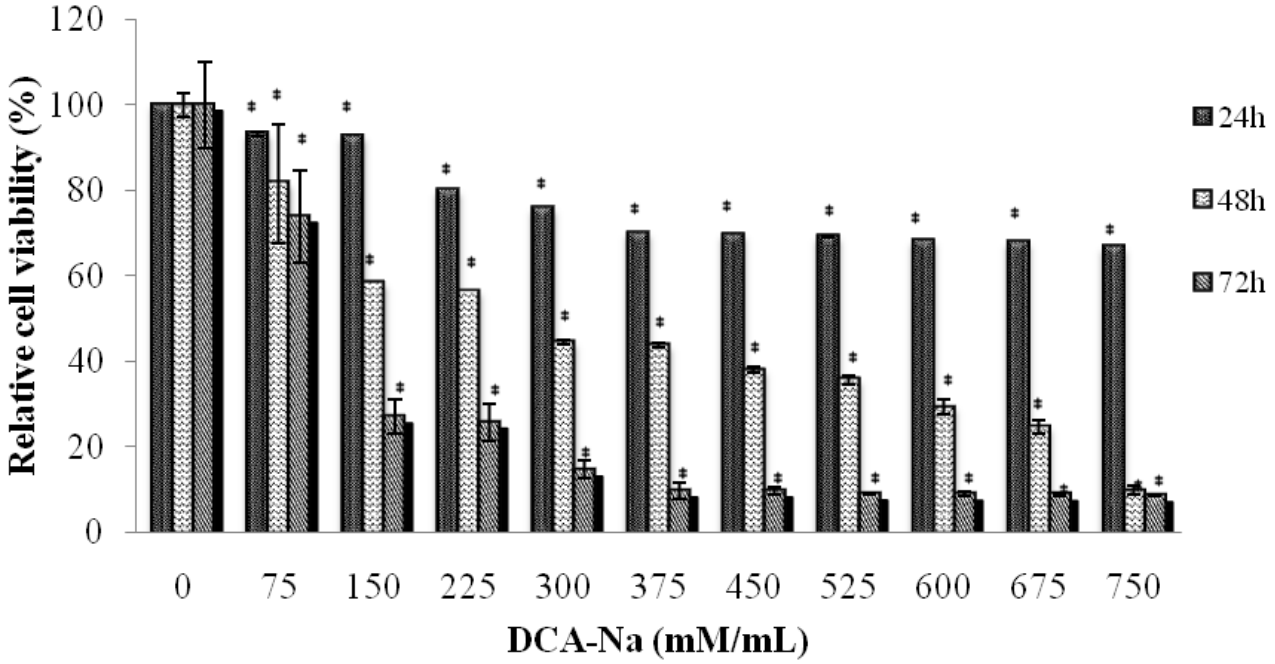

Figure 3. Cell viability of B16F10 cell line treated with DCA-Na. B16F10 cells (5×10^3 cells/well) were cultured into 96 well plates and incubated overnight. Thereafter, the plates were treated with DCA-Na concentrations ranging from 0 to 750 mM/ml, and incubated for 24, 48, and 72 h at 37°C, and 5% CO_2 atmosphere. Thereafter, a standard MTT assay was performed. The optical density was determined at 570 nm. Data represent means of triplicate samples with ±SD indicated. *p<0.05 as compared with untreated cells.

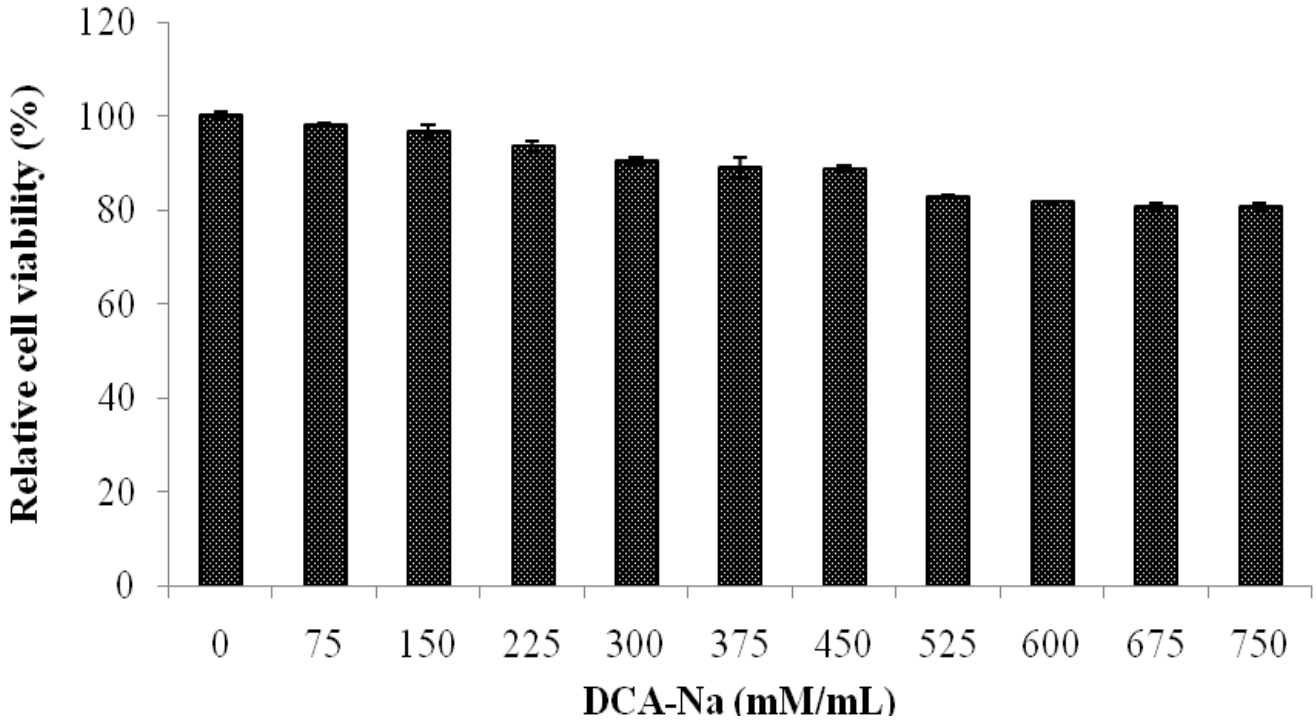

Figure 4. Cell viability of macrophages treated with DCA-Na. Murine peritoneal macrophages from 6-8 weeks old female C57BL/6 mice were harvested and cultured into 96-well plates (5×10^3 cells/well), and incubated overnight. Thereafter, the plates were treated with DCA-Na concentrations ranging from 0 to 750 mM/ml, and incubated for 72 h at 37°C, and 5% CO_2 atmosphere. Thereafter, a standard MTT assay was performed. The optical density was determined at 570 nm. Data represent means of triplicate samples with ±SD indicated. *p<0.05 as compared with untreated cells.

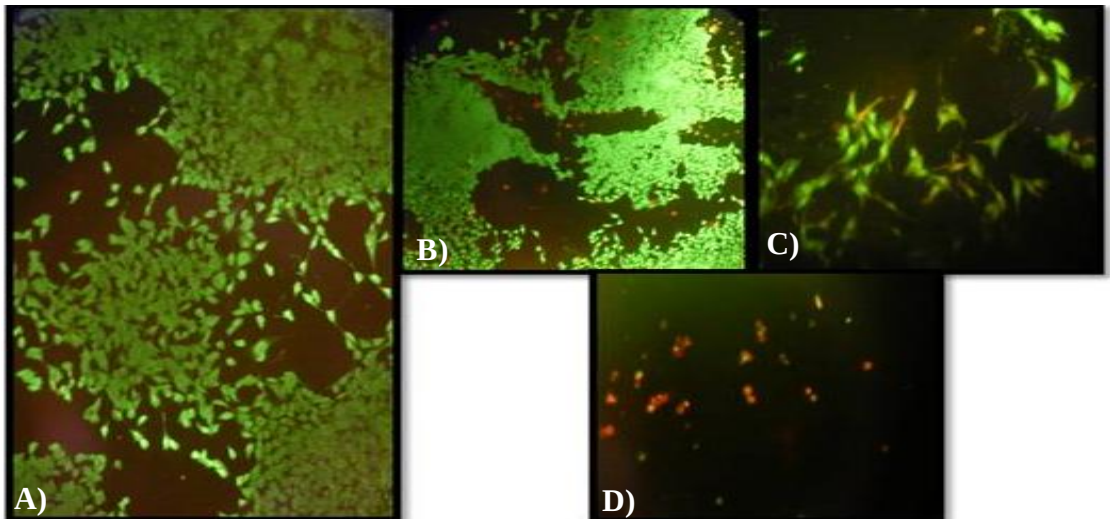

Figure 5. Determination of apoptosis by acridine orange-ethidium bromide staining in B16F10 cell line treated with DCA-Na. B16F10 cells (1×10^6 cells/well) were treated with LD_{20}, LD_{50}, and LD_{90} of DCA-Na for 48 h. After one washing with PBS, the cells were stained with 2 µL of a mixture (1:1) of acridine orange-ethidium bromide in PBS. The cells were incubated for five minutes in the dark at room temperature, and then were viewed under a Nikon inverted fluorescent microscope. Detection of apoptosis was based on morphological and fluorescent characteristics of the stained cells. Viable cells were indicated by bright green color, apoptotic cells by orange/brown color, and necrotic cells by red color. Each experiment was done in triplicate and repeated at least three times. A) untreated B16F10 cells, B) LD_{20}, C) LD_{50}, and D) LD_{90}.

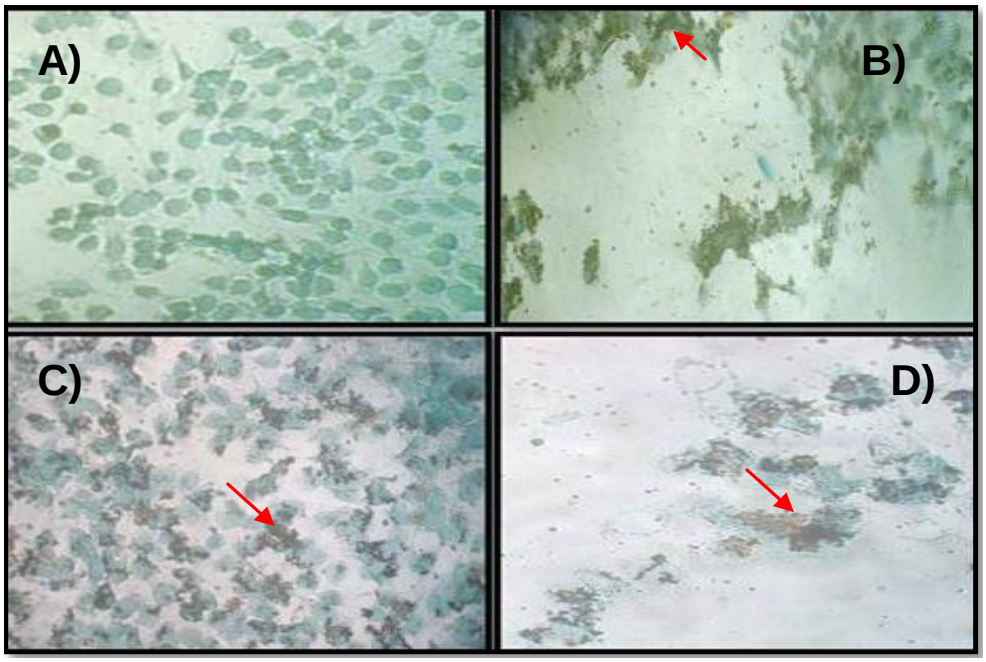

Figure 6. Analysis of apoptosis by TUNEL, in B16F10 cell line treated with DCA-Na. After DCA-Na treatment (LD_{50} and LD_{90}, by 48 h) on B16F10 cells (5×10^3 cells/well), the cells were digested with proteinase K (20 µg/ml). TdT and biotinylated dNTP in buffer were then added to cover the cells and incubated in a humid atmosphere at 37°C for 60 minutes. The cells were washed with PBS and incubated with streptavidin-horseradish peroxidase for 10 minutes. After rinsing with PBS, the cells were immersed in DAB solution. The cells were counterstained for 3 minutes with 1% methyl green. Cells containing fragmented nuclear chromatin characteristic of apoptosis will exhibit brown nuclear staining that may be very dark after labeling and photographs were taken. a) in the upper left square , b) in the upper right square, c) in the lower left cuadrant, d) in the lower right cuadrant.

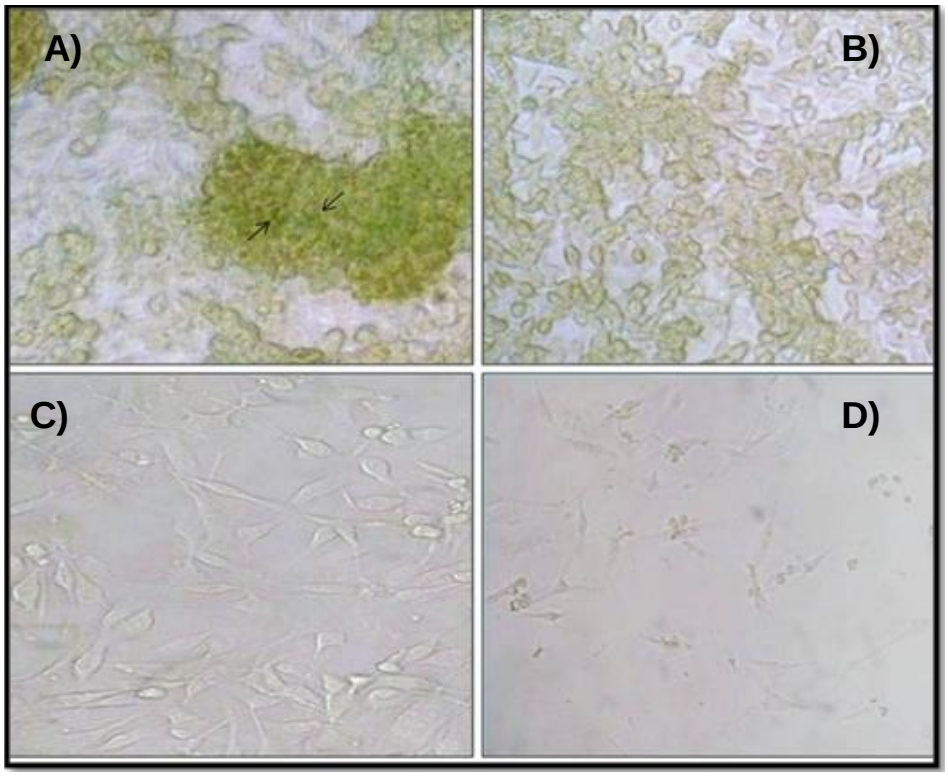

Figure 7. Effect of DCA-Na on cellular senescence in B16F10 cell line. B16F10 cells (1×10^6 cells/well) were treated with LD_{50} and LD_{90} of DCA-Na for 48 h. Doxorubicin was used as a positive control (0.005 µg/ml). Cells then were rinsed with PBS, and 100 to 200 cells in six microscopic fields were counted to determine the percentages of SA-β gal stained positive cells. a) in the upper left square , b) in the upper right square, c) in the lower left cuadrant, d) in the lower right cuadrant.

process and is overexpressed in several cancer cell lines and tumors, when the B16F10 cells were treated with DCA-Na at doses of LD_{20}, LD_{50}, and LD_{90}, the GAPDH gene expression was decreased (similar to 18S and β-actin housekeeping genes) (Figure 10) and also was observed a decrement of LDH activity (is an enzyme involved in the conversion of pyruvate to lactate) in a dose dependent manner since 24 h of treatment (*$p<0.05$) but not change was observed at 48 h (Figure 8). The evaluation of cAMP-PKA activity in the B16F10 cells without treatment shown phosphorylated and non-phosphorylated forms, and the treatment of DCA-Na at doses of LD_{20}, LD_{50}, and LD_{90} inhibited the cAMP-PKA complex phosphorylation at 48 h (Figure 9).

DNA binding-activity of HIF-1α

HIF regulates a large panel of genes involved in angiogenesis, glucose regulation (glucose transporter-1, phosphoglucokinase-1), and NO signaling (Duyndam et al., 2001). In the B16F10 cells, the DNA binding-activity of HIF-1α was significantly decreased (*$p<0.05$) by the treatments with DCA-Na (LD_{50} and LD_{90}) compared with the B16F10 cells without treatment that had an increased DNA binding-activity of HIF-1α (Figure 11).

DCA-Na reduced the melanoma invasion

We used a Boyden chamber assay to quantify invasion into reconstituted basement membrane (Matrigel). B16F10 cells shown a highly power of invasion, and the treatments with DCA-Na significantly reduced the capacity of cellular invasion, in a dose and time dependent manner to approximately 60 and 87.5% at doses of LD_{50} and LD_{90} at 24 h, respectively; and 75 and 92.5% at doses of LD_{50} and LD_{90} at 48h, respectively (Figure 13 and Table 1, *$p<0.05$).

Effect of DCA-Na on angiogenesis

To evaluate the capacity of DCA-Na at doses of LD_{50} and LD_{90} on angiogenesis, we used an angiogenesis inductor control (PMA 1 mM) and angiogenesis inhibitor control (JNJ 3 mM) provided for the manufacturer. We observed that DCA-Na inhibited the formation of pro-angiogenic

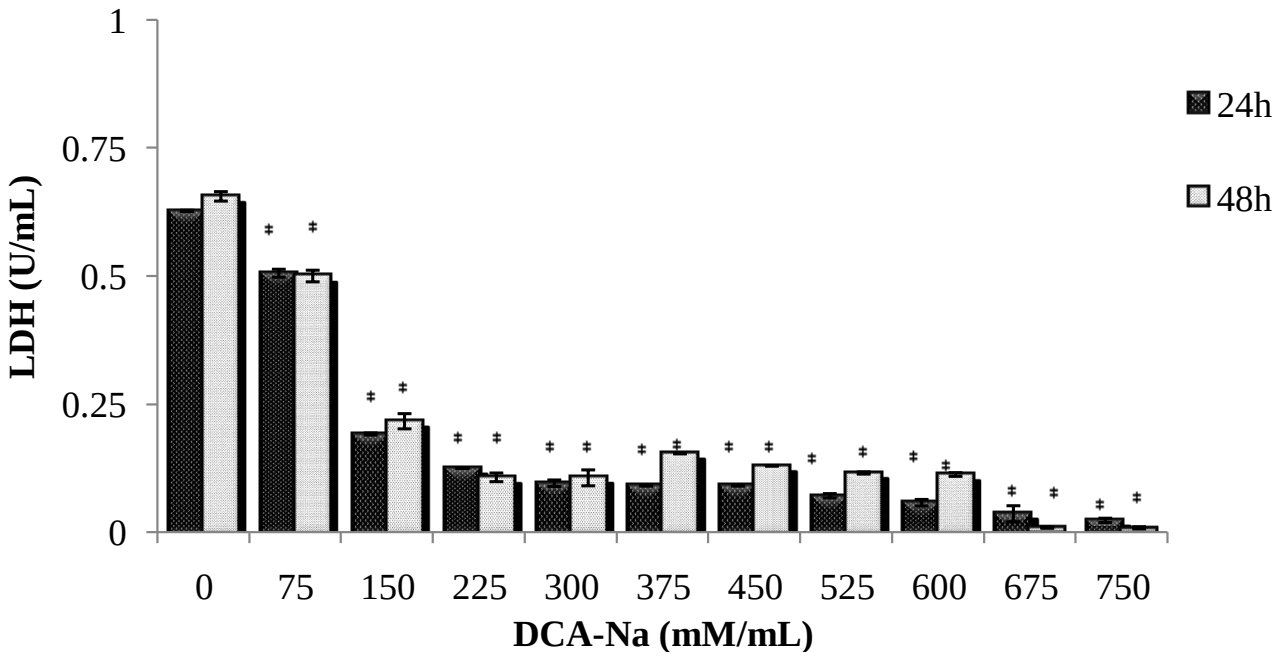

Figure 8. Effect of DCA-Na on LDH activity in B16F10 cell line. B16F10 cells (5×10^3 cells/well) were cultured into 96 well plates and incubated at 37°C in 5% CO_2 atmosphere with DCA-Na at concentrations ranging from 0 to 750 mM/ml. The plates were then incubated for 24 and 48 h at 37°C, and 5% CO_2 atmosphere. Thereafter, LDH activity was measured by changes in optical densities due to NAD^+ reduction which was monitored at 490 nm. The experiments were performed in triplicates; data shown represent mean ±SD of three independent experiments. *p<0.05 as compared with untreated cells.

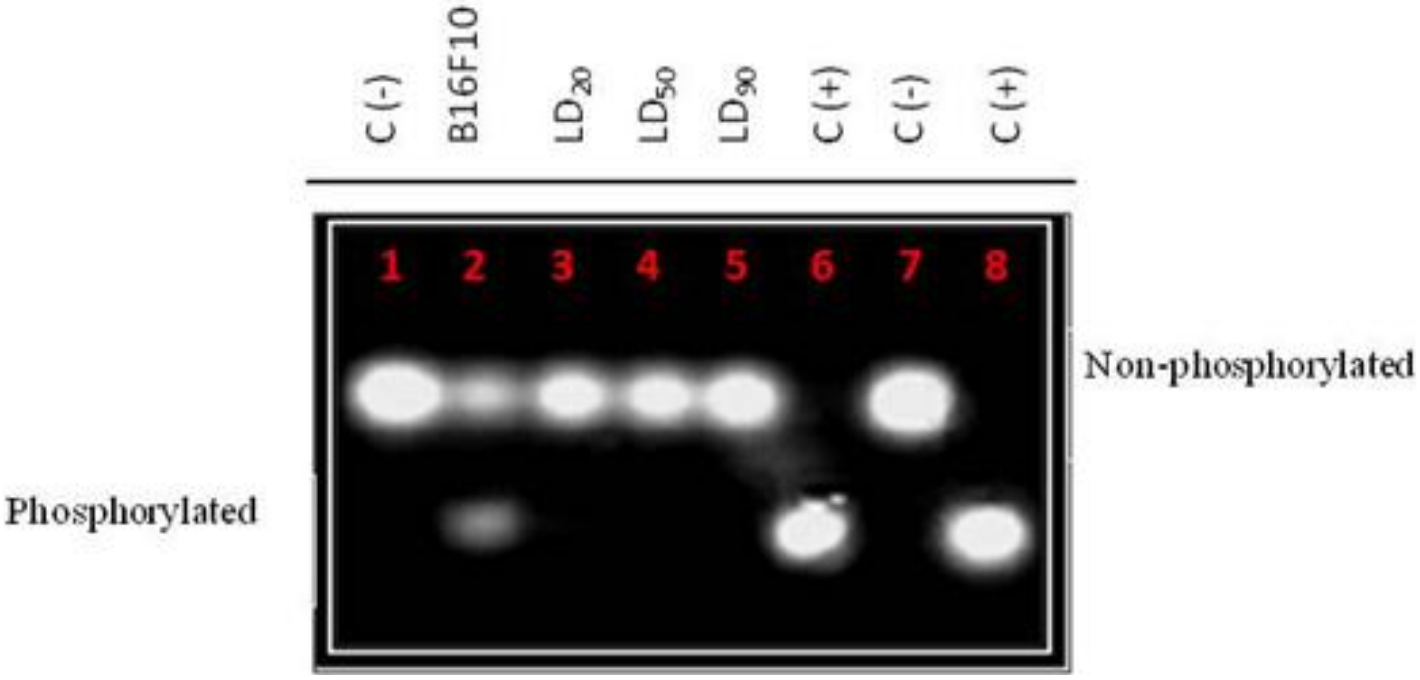

Figure 9. Effect of DCA-Na on cAMP dependent protein kinase A activity in B16F10 cell line. The B16F10 cells (5×10^6 cells/T25 flasks) were treated with LD_{20}, LD_{50}, and LD_{90} of DCA-Na for 48 h. Thereafter, the cells were harvested and crude cell extracts were prepared and 2 mg of Pep Tag peptide substrate were incubated with cell lysates for 10 min at room temperature. The reaction was stopped by heating at 95°C for 10 min and the samples were resolved on a 0.8% agarose gel with phosphorylated peptide moved toward the anode (Lanes 6 and 8) and non-phosphorylated peptide moved toward the cathode (Lanes 1, 3, 4, 5, and 7). The B16F10 cancer cells shown forms phosphorylated and non-phosphorylated. C (-): PKA activator, C (+): PKA activator with cAMP-dependent protein kinase, catalytic subunit. This experiment was conducted three times with similar results.

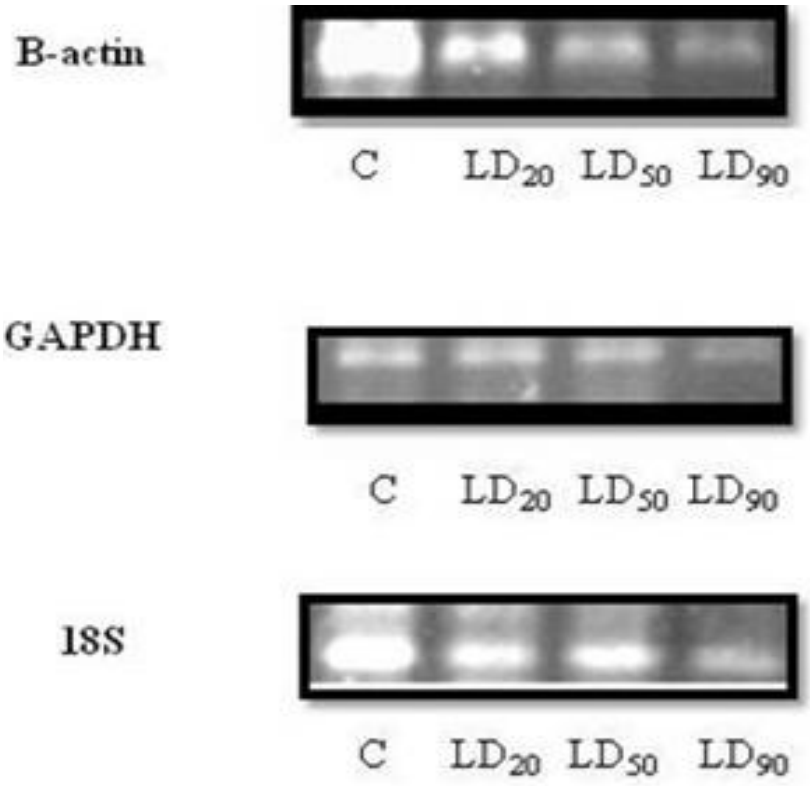

Figure 10. Effect of DCA-Na on GAPDH β-actin, and 18S expression in B16F10 cell line. Total RNA was extracted from 1×10^6 B16F10 cells untreated (C) and B16F10 cells treated with DCA-Na at doses LD_{20}, LD_{50}, and LD_{90} for 48 h. The expression of β-actin, GAPDH, and 18S were performed by RT-PCR. The size of PCR products were analyzed by electrophoresis in 1.2% agarose gels and visualized by ethidium bromide staining with UV light and photographed.

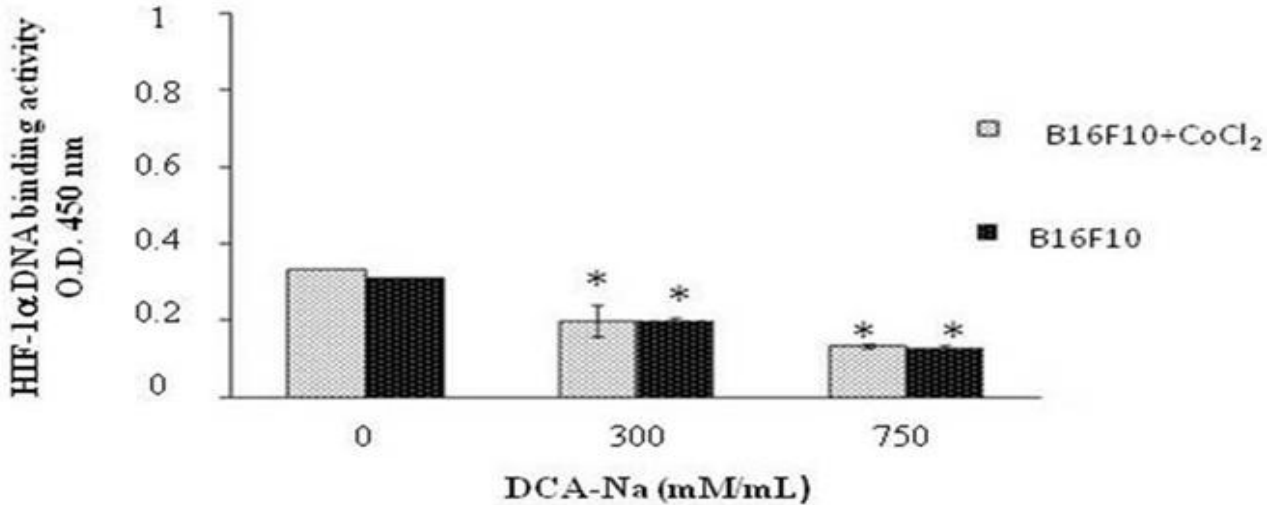

Figure 11. Effect of DCA-Na on HIF-1α DNA binding activity in B16F10 cell line. B16F10 (1×10^7cells/T75 flasks) with or without hypoxia inducted by $CoCl_2$, were treated with LD_{50} and LD_{90} of DCA-Na for 48 h of incubation at 37°C in 5% CO_2 atmosphere, cell-free supernatants were isolated following centrifugation at 600×*g* for 10 min. HIF-1α was measured in the supernatants using a commercial ELISA kit (HIF-1α, Transcription Factor Assay Cayman Chemical kit, USA) according to the manufacturer's instructions. The reactions were stopped by the addition of 100 µL of Stop Solution. The HIF-1α DNA binding activity was measured by absorbance readings (450 nm). *p<0.05 as compared with untreated cells.

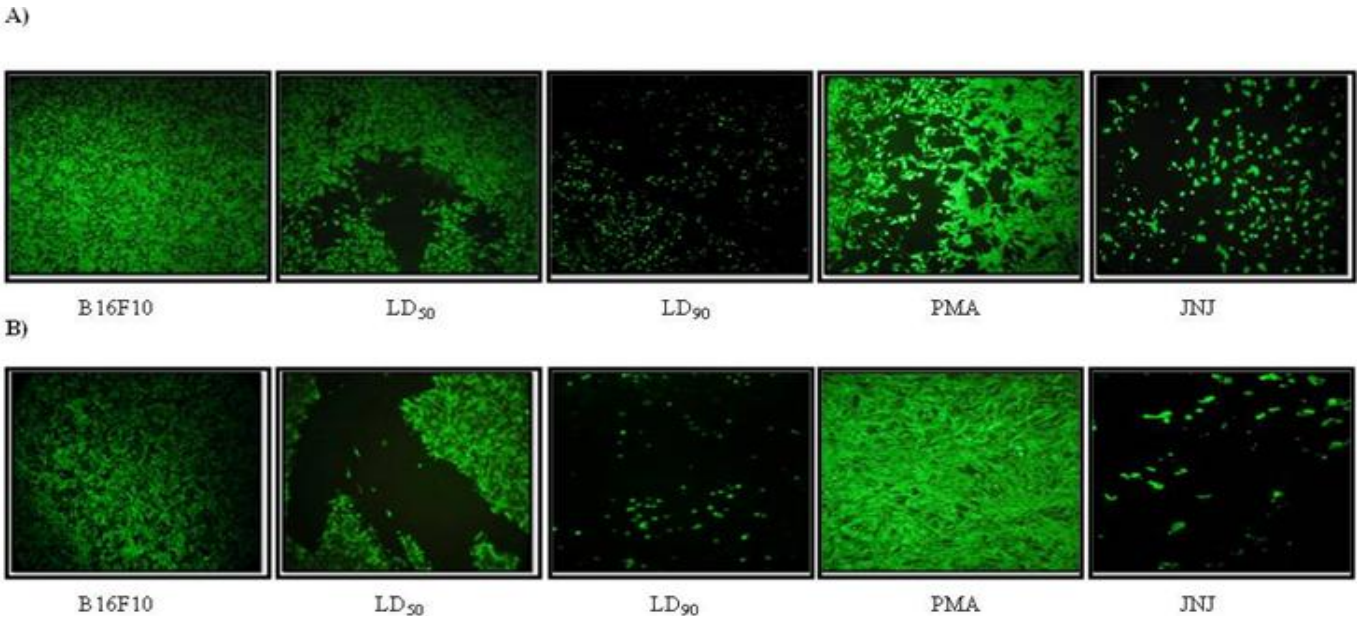

Figure 12. Effect of DCA-Na on angiogenesis in B16F10 cell line. Forty microlitre of cell-based extracellular matrix gel per well was added in 96-well tissue culture plates and incubated for 1 h at 37°C to solidify, after 5×10^3 B16F10 cells/well were plated and allowed to attached overnight and thereafter the cells were treated with LD_{50} or LD_{90} of DCA-Na, or PMA 1mM (angiogenesis stimulation) or JNJ 1mM (angiogenesis inhibition) and incubated at 37°C in 5% CO_2 atmosphere for A) 24 and B) 48 h. After 10 µL of fluorescent dye Calcein AM solution (1:100) per well was added for visualization of cellular organization by fluorescence microscopy (Nikon inverted fluorescent microscope, TE-Eclipse 300).

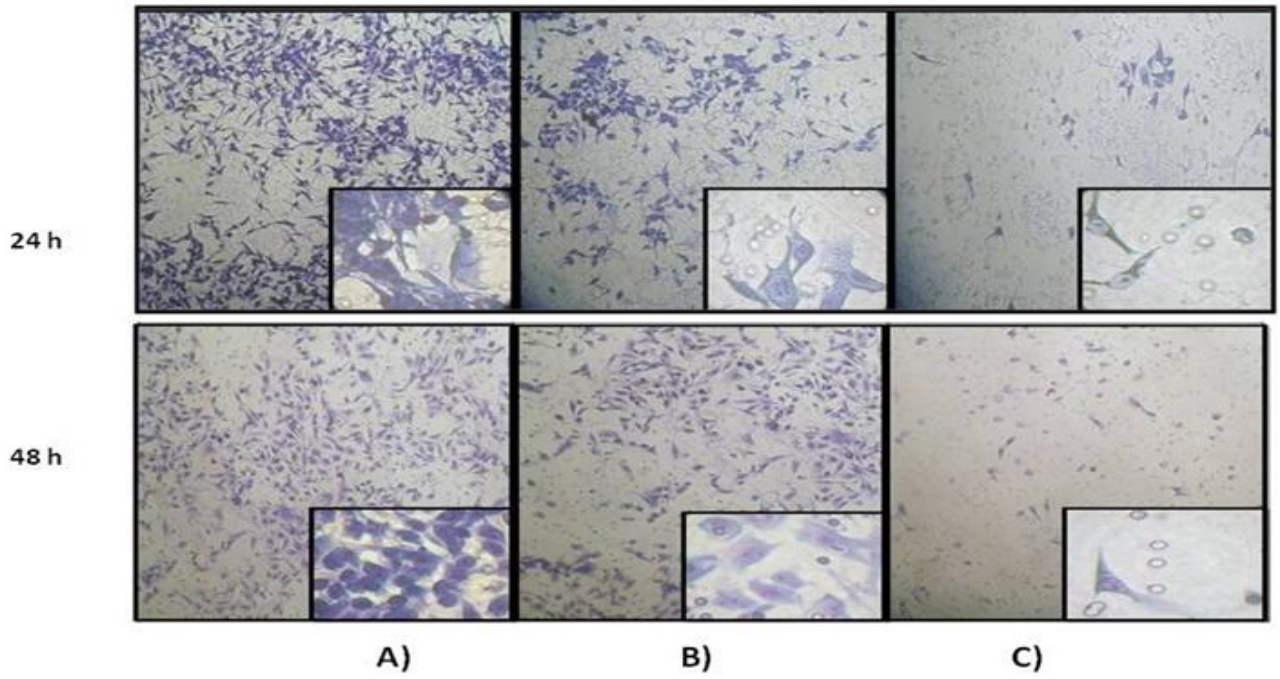

Figure 13. Effect of DCA-Na on invasion in B16F10 cell line. B16F10 cells (1×10^6 cells/well) were treated with LD_{50} and LD_{90} of DCA-Na for 22 and 48 h of incubation at 37°C in 5% CO_2 atmosphere. Matrigel Invasion Chamber for induced chemoattraction 2.5 ml of serum fetal bovine at 5% was added to each well and immediately covered with the Cell Culture Inserts containing an 8 µ pore size PET membrane with a thin layer of MATRIGEL Basement Membrane Matrix. The layer occludes the pores of the membrane, blocking non-invasive cells from migrating through the membrane. In contrast, invasive cells (malignant and non-malignant) are able to detach themselves from and invade through the Matrigel Matrix and the 8 µ membrane pores. A) untreated B16F10 cells, B) LD50, and C) LD90 of DCA-Na.

Table 1. Effect of DCA-Na on B16F10 cellular invasion.

Invasion (%)			
Time (h)	**Control**	**LD_{50}**	**LD_{90}**
22	100	40*	12.5*
48	100	25*	7.5*

B16F10 cells (1x106) were treated with LD50 and LD90 of DCA-Na for 22 and 48 h of incubation at 37°C in 5% CO_2 atmosphere. The percentage invasion was determined by means of cells invading through Matrigel insert membrane / means of cells migrating through control insert membrane. *p<0.05 compared to the controls.

Table 2. Comparison of volume-weight of tumors in mice treated with DCA-Na.

Treatments	Body weight (g±SD)	Tumor weight (g±SD)	Tumor volumen (mm^3)
Control	21.15±1.84	8.16±0.21	21.09±2.05
DCA-Na (500 mg/kg)	22.53±2.6	1.74±0.63*	2.35±0.17*

C57BL/6 mice bearing B16F10 tumor were treated with DCA-Na at doses of 500 mg/kg/ twice per day, injected by intraperitoneal route. In this study the mice were sacrificed at 28 days, tumor weight and tumor volume were measured every 7 days as described in materials and methods. *p<0.05 compared to the controls.

Table 3. Time to appearance of tumor, death, and survival mice treated with DCA-Na.

Treatments	Mean time to appearance of tumor (days±SD)	Mean time of death mice (days±SD)	Death (%)	Survival (%)
NI	0	0	0	100
Control	11.5±2.88	30.2±1.92	100	0
DCA-Na (500mg/kg)	11.5±2.88	31.66±0.47	80	20
DCA-Na (1000mg/kg)	11.5±2.88	24.33±3.51	100	0

Groups of C57BL/6 mice were treated with DCA-Na at doses of 500 or 1000 mg/kg/ twice per day, injected by intraperitoneal route. The mice were monitored until death or recovery. *p <0.05 compared to the control. NI: non-inoculated.

structures in a dose dependent manner at 24 and 48 h of treatment similar to JNJ treatment (Figure 12).

Effect of DCA-Na on mortality, tumor weight and diameter, and metastasis

When used the DCA-Na (500 mg/kg/twice per day, injected by intraperitoneal route), the mice did not delay the death time (30.2±1.92 days) compared with control mice (31.66±0.47 days), but the treatment induced survival of 20% in mice (Table 3 and Figure 14). In mice sacrificed at 28 days, the DCA-Na treatment significantly decreased the tumor weight and diameter (1.74 g and 2.35 mm^3, respectively), compared with control (8.16 g y 21.09 mm^3, respectively) (*p<0.05) (Table 2). At necropsy, metastasis was not observed in mice treated with DCA-Na compared with control mice (metastasis in peritoneal cavity, bowel, and liver) (Table 4). When used 1000 mg/kg/twice per day, mice death was observed at 24.33±3.51 days (Table 3).

DISCUSSION

The effect of DCA-Na on diminished the cellular viability of different cancer cell lines (TC-1, Hela, PC3, DU145, Huh-7, Iner 51, MDAMB-231, MCF-7, and B16F10) without affected the viability of macrophages *in vitro*, are similar to obtained by Wong et al. (2008), on endometrial (AN3CA, SKUT1B, RL95-2, and KLE) and breast (MCF-7) cancer cell lines and by Madhok et al. (2010) on colorectal cancer (SW480, HT29) and by Sun et al. (2010) on MCF-7, T-47D, 13762MAT and V14 cancer cell lines. The growth of several tumors is related to cAMP/PKA pathway, because this complex can activate different proteins involved in the signal transduction and cellular growth (Caretta and Mucignat, 2011). In our study, the fact that DCA-Na, diminish the activity of cAMP/PKA complex, and inhibit the LDH activity in B16F10 cancer cells, could indicate the capacity of this agent to induce the regression of this cells to glycolytic phenotype causing the cellular death. This data are correlated in part with Sun et al. (2010), where the DCA-

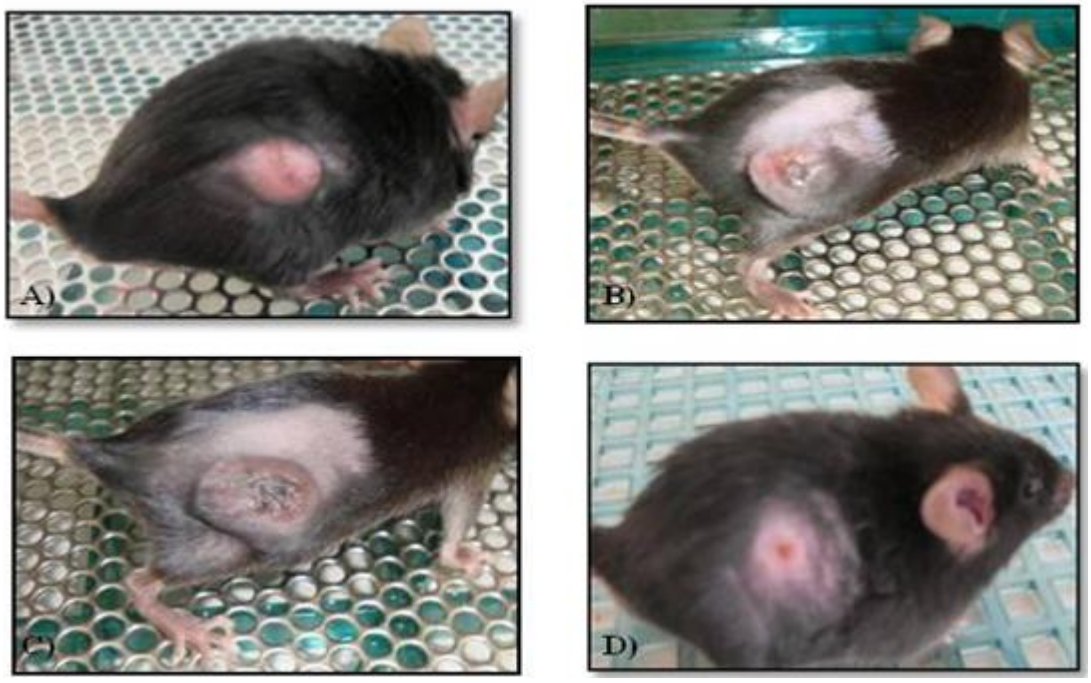

Figure 14. Decreased tumor growth in mice treated with DCA-Na. C57BL/6 mice bearing B16F10 tumor were treated with DCA-Na at doses of 500 mg/kg/ twice per day, injected by intraperitoneal route and the treatment decreased the tumor growth, inducing tumor cellular death focus in comparison with control. A) mouse bearing with B16F10 tumor (11.5 days tumor growth), B) mouse bearing with B16F10 tumor at 7.5 days of DCA-Na treatment shown a tumoral death focus, C) mouse bearing with B16F10 tumor at 11 days of DCA-Na treatment shown and increasing in tumoral death focus, and D) mouse bearing without tumor after 21 days of treatment with DCA-Na. These photos are representative of mice groups with and without treatment.

Table 4. Incidence of metastasis in mice treated with DCA-Na.

Treatments	Muscle	Peritoneal cavity	Bowel	Liver
Control	✔	✔	✔	✔
DCA-Na	✔			

C57BL/6 mice bearing B16F10 tumor, were treated with DCA-Na at doses of 500 mg/kg/ twice per day, injected by intraperitoneal route. In this study the mice were sacrificed by cervical dislocation at 28 days; and the metastasis was determined during the necropsy in muscle, peritoneal cavity, bowel, and liver.

Na treatment in the MAT breast cancer cell, diminish the concentration of extracellular lactate. This results are important because LDH are overexpressed and participated in the growth and maintaining tumoral, controlling the conversion of pyruvate to lactate in the cellular glycolytic process (Fantin et al., 2006). The expression of LDH is regulated by HIF-1α, and when is activated begin the genes transcription associated to invasion and metastasis (Xu et al., 2005). In hypoxia conditions, the HIF-1α subunit is stabilized and translocated to the nucleus where dimerizes with HIF-1α and by union to response elements to hypoxia active the transcription of several genes that participate in key steps of tumorigenesis, included angiogenesis, metabolism, proliferation, metastasis, and differentiation (Semenza, 2008). Ours results shown that DCA-Na treatment on B16F10 cells, has the potential to diminish the HIF-1α DNA binding activity, cellular invasion capacity and angiogenesis, possibly affecting the percentage of cellular invasion, avoiding the angiogenesis formation (Duyndam et al., 2001). Hypoxia is a decrease in the oxygen levels in the tissues, is a characteristic of tumor microenvironment, and has been important by its contribution in increase the tumorigenicity of cancer cells by selection of more metastatic and aggressive clones and by pathways that contribute to survival of cancer cells, with this data, a hypothesis can be done suggesting that HIF-1α and VEGF are implicated in the intratumoral

hypoxia could be a target of treatment in solid tumors independent of their genetic alterations (Rapisarda et al., 2009). GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) is well known for its glycolytic function of converting GAPDH to 1,3-bisphosphoglycerate in the cell and has been shown to be up-regulated in many cancers and down-regulated by chemotherapy drugs (Valenti et al., 2006). The DCA-Na (LD_{90}), affected the GAPDH and housekeeping genes expression β-actin and 18S), being difficult their normalization. Suggesting the capacity of DCA-Na in affect energetic pathways (GAPDH) (Lee et al., 2011), structural and functional components of cells (β-actin) (Schmittgen and Zakrajsek, 2000), and the machinery of proteins translation (18S) in the B16F10 cells (White, 2008); furthermore, although several references (Lee et al., 2011; Schmittgen and Zakrajsek, 2000; White, 2008) suggesting the use of the ribosomal RNA 18S, β-actin, and GAPDH to normalization, we have not recommended the use of these housekeeping genes as a control, in particular in studies investigating the effects of DCA-Na on B16F10 cancer cells, seems to be inappropriate and RT-PCR data on the effects of DCA-Na in cancer cells should be reviewed by a quantitative approach using real time PCR, with a different housekeeping gene (Valenti et al., 2006). The cell death induced by apoptosis and not generation of cellular senescence seems to be the principal determinant of mortality in the B16F10 cancer cells treated with DCA-Na. Shahrzad et al. (2010), shown similar data when cancer colorectal cell lines (SW480 and Caco-2) were treated with DCA in normoxic conditions conducted to death by apoptosis, but the apoptosis induction decreased when this cells were treated in hypoxic conditions, possibly through HIF-1alpha dependent pathways. Although the senescence and cell death are safe mechanisms against tumorigenesis, both forms of cellular response could be induced in the cancer cells, suppressing the tumoral progression (Litwiniec et al., 2010). By other way, the effect of administration of DCA-Na in mice with tumor, induced in our study a survival of 20%, decreased of tumoral weight, diameter and volume**,** correlated in part with another study by Bonet et al. (2009), where DCA-Na treatment decreased the tumoral growth. Characteristically the DCA-Na treatment induce at the 7.5 ± 1.84 days in the tumor a cell death focus in the tumor center, in some mice this lesion begin to advance until the tumor eradication and in another mice, this lesion is maintained although the tumor is not eliminated. The results of this study demonstrated that DCA-Na had the capacity to *"In vitro"* decreased the cellular viability, induced cellular death by apoptosis, affected different metabolic pathways (LDH, cAMP/PKA, HIF-1α, and GAPDH), decreased the cellular invasion and angiogenesis; and *"In vivo"* diminished the tumoral diameter, volume and weight, without affected the body weight and avoided metastasis in C57BL/6 mice, these findings could explain in part some mechanism of DCA-Na on B16F10 cells but more studies are necessary to be conducted.

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