Advances in Cancer Research & Therapy

Anveshika
Publishing & Informatics

RESEARCH ARTICLE

Metabolic synergy of breast cancer cells with tumor microenvironment: Interaction of breast cancer cells with normal fibroblasts, cancer-associated fibroblasts and adipocytes in vitro

Cheppail Ramachanndran, [1,2*], Ashley Juan[2], Daniel Moy [2], Karl-Werner Quirin[3] Enrique Escalon, [4], Ziad Khatib[4] and Steven J. Melnick[1,2]

[1]*Department of Pathology, Nicklaus Children's Hospital, Miami, FL 33155*
[2]*Dharma Biomedical LLC, Miami, FL 33156*
[3]*Flavex Naturextrakte GmbH, Rehlingen, Germany*
[4]*Division of Hematology/Oncology, Nicklaus Children's Hospital, Miami, FL 33155*

ABSTRACT

Tumor microenvironment (TME) plays an important role on the progression and metastasis of tumors because of metabolic coupling and interaction between non-malignant cells in the TME and cancer cells. We have investigated the role of cancer-associated fibroblasts (CAF), normal fibroblasts (NF) and adipocytes on the growth of breast carcinoma cells in vitro. Additionally, the anti-glycolytic effects of supercritical CO_2 extract of mango ginger (Curcuma amada Roxb) (CA) on both conventional and reverse Warburg effects were studied in the monocultures of CAF (CAF-05), NF (CCL-110), adipocytes (HPAd) and breast cancer (MCF-7) cells and co-cultures of breast cancer cells with CAFs, NF and HPAd. CA was highly cytotoxic to CAF-05 and MCF-7 cell lines compared to CCL-110 and HPAd cell lines. CA inhibited ATP synthesis in monocultures and co-cultures, though the rate of inhibition is higher in co-cultures than MCF-7 monoculture. Although non-malignant cell lines (CAF-05, CCL-110 and HPAd) have lower levels of lactate synthesis than MCF-7 cells, inhibitory effect of CA is more pronounced in co-cultures of MCF-7 with CAF-05, CCL110 and HPAd cells, respectively than MCF-7 cells alone. Gene expression studies showed that although transcripts of glycolysis-associated genes HIF-1α, MCT1, MCT4, Caveolin-1 and GLUT1 were present in non-malignant monocultures, protein translation of these genes were absent. Treatment of co-cultures of MCF-7+CAF-05 and MCF-7+HPAd cells with supercritical CO_2 extract of mango ginger [Curcuma amada Roxb. (CA) - 5 g/ml] down regulated MCT1, MCT4, and Caveolin-1 proteins. CA treatment also inhibited HIF-1α and GLUT1 protein expression in co-cultures of MCF-7+HPAd cells. These results indicate that CA inhibits both Warburg and reverse Warburg effects in TME.

KEYWORDS

ATP, adenosine triphosphate; CA, supercritical CO_2 extract of mango ginger; CAA, cancer associated adipocytes; CAF, cancer associated fibroblasts; CAV-1, Caveolin-1, DMEM, Dulbecco's modified eagle medium; FBS, fetal bovine serum; GLUT1, glucose transporter 1; HIF-1α, hypoxia inducible factor-1α; HPAd, human pre-adipocyte; LDD, E-labda-8(17),12diene-15,16 dial; MCT, mono-carboxylate transporter; MTT, 3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF, normal fibroblasts; PI3K, phospho-inositol-3 kinase; ROS, reactive oxygen species; SDS-PAGE, sodim dodecyl sulfate-polyacrylamide gel electrophoresis

HISTORY
Received :23 April 2019
Accepted :02 January 2020
Published :18 January 2020

Volume - 4 Issue - 1

1. INTRODUCTION

Otto Warburg (1956) demonstrated that metabolism of tumor cells differs from normal cell metabolism due to the preferential use of aerobic glycolysis (Warburg effect) for the production of energy in the form of adenosine triphosphate (ATP). Furthermore, enhanced glycolysis and high lactate production are important hallmarks of tumor progression owing mainly to the Warburg effect (Warburg and Wind 1927; Warburg 1956; Hanahan and Weinberg 2011; Gentric et al. 2017). The Warburg effect involves the catabolism of glucose to lactate, a high energy metabolite, for producing ATP. Once produced, lactate can then be transferred to other cells that are undergoing oxidative mitochondrial metabolism (oxidative phosphorylation). This intracellular transport of lactate occurs via specialized transporters known as mono-carboxylate transporters (MCTs). Moreover, the Warburg effect in cancer cells and the hypoxic state that is produced are supported by a variety of metabolic regulators and signaling pathways i.e., phospho-inositol-3 kinase (PI3K), hypoxia-inducible factor-1 (HIF-1), and c-MYC signaling (Diedrich et al. 2015). Cancer associated fibroblasts (CAFs) are a group of specialized fibroblasts considered to be the principal non-malignant stromal cell type within the tumor microenvironment (TME). Recent research focused on the role of CAFs in the progression and metastasis of cancer has demonstrated a dynamic interaction between stroma and epithelium (Bonnans et al. 2014; Costa et al. 2014; Karangiannis et al. 2012; Ohlund et al. 2014). While normal fibroblasts inhibit tumor progression, transformation of fibroblasts to a CAF phenotype and other alterations of the extratracellular matrix potentially produce a conducive environment for carcinogenesis (Bissel et al 2011; Costa et al 2014). CAFs have been shown to promote

Address for Correspondence: Cheppail Ramachandran Ph.D., MBA, Sr. Research Scientist and Chief Science Officer, Nicklaus Children's Hospital, Miami Children's Health System, Miami, FL 33155. Phone: 305-663-8510; Email: cheppail.ramachandran@mch.com

proliferation, and migration of tumor cells and other hallmarks of cancer; angiogenesis and metastasis through remodeling of the extracellular matrix and epithelial-to-mesenchymal transition. The hypoxic environment created by the tumor, induces the reverse Warburg effect (anaerobic glycolysis in stromal cells) in the CAFs that through the upregulation of signaling pathways such as HIF-1α-signaling increases glycolysis and the resulting generation of nutrients such as lactate to adjacent cancer cells (reverse Warburg effect) (Pavlides et al. 2009).

Metabolic coupling between glycolytic stromal cells (including CAFs) and oxidative cancer cells and between glycolytic cancer cells (further from the vascular supply) and oxidative cancer cells promote tumor growth in human breast and other cancers (Rattigan et al. 2012; Gupta et al. 2017; Martinez-Outschoorn et al. 2017). The TME plays an important role for growth and progression of breast cancers since non-malignant cells of the stroma provide tumor promoting molecules to these tumors. Although adipocytes are also a major component of the TME of mammary tumors, cross talk of breast cancer cells with the surrounding adipocytes has not been well-investigated. During early tumor invasion, breast cancer cells invade the mammary fat pad in direct juxtaposition with adjacent adipocytes (Dirat et al. 2011). These cancer associated adipocytes (CAA) secrete adipokines, growth factors, proteases and fatty acids analogous to the role of CAFs in promotion of tumor growth and survival (Hoy et al. 2017; Wang et al. 2012). Currently, little is known about how adipocyte-derived lipids directly influence tumor metabolism; however, there is increasing evidence that lipids generated during lipogenesis modulate metabolic pathways in cancers cells and stimulate Warburg effect (Diedrich et al 2015; Blucher and Stadler 2015). Adipocytes, especially those that comprise white fat versus brown fat, are predominantly glycolytic even in an aerobic environment and thus produce large amounts of lactate (DiGirolamo et al. 1992; Sabter et al. 2014; Jansson et al. 1994). Although these adipocytes have the ability to influence the neighboring cells within their microenvironment, the role of adipocytes in regulating tumor metabolism is less clear. The cancer promoting effects of adipocytes on growth, proliferation and survival of tumors has been indicated in breast, prostate, gastric, colon and ovarian cancers (Nieman 2011; Nieman et al. 2013); however, little is known about their contribution to the alteration of tumor metabolism. We have investigated the role of normal fibroblasts (NF), CAFs and adipocytes on the growth and proliferation of breast cancer cells and also the underlying molecular and metabolic alterations in vitro. The inhibitory effect of supercritical CO_2 extract of mango ginger (Curcuma amada Roxb.) on the Warburg effect in these cell types was also analyzed at the biochemical and molecular levels.

2 .Materials and Methods

2.1 Cell Culture

Human breast carcinoma (MCF-7) and normal fibroblast (CCL-110) cells were purchased from American Type Culture Collection, Manassas, VA and the cells were grown in Dulbecco's modified eagle medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS) and antibiotics in a humidified 5% CO_2 incubator. Human breast cancer-associated fibroblast (CAF-05) and human pre-adipocyte (HPAd) cell lines were purchased from Neuromics (Edina, MN) and Sigma-Aldrich (St.Louis, MO), respectively. CAF-05 cell lines were cultured in media supplied by Neuromics where as HPAd cell line was grown in pre-adipocyte cell culture medium (Sigma-Aldrich, St. Louis, MO). HPAd cell lines were differentiated before drug treatment of monocultures and co-cultures (Petersen et al. 2017) by growing the cells first to confluency in DMEM, and 1-2 day post-confluent cells (designated day 0) were induced to differentiate by adding DMEM containing 1 M dexamethasone, 0.5 mM methyl isobutyl xanthine, 5 g/ml insulin/transferrin/selenium (I/T/S) solution (Life technologies, Inc. and 0.5 M rosiglitazone (Sigma-Aldrich, St. Louis, MO).

2.2 Supercritical CO_2 extract of mango ginger (Curcuma amada Roxb.)

Supercritical CO_2 extract of mango ginger (CA) was prepared by Flavex Naturextrakte GmbH, Rehlingen, Germany. The usual yield of CA extract was 2.5-3% of dried rhizome. The product is brownish and contains 10.2% of steam volatile components. Quantitative analysis by HPLC and GC-MS showed the presence of 61.7% (E)-labda-8(17),12diene-15,16 dial (LDD), 5.6% beta myrcene, 0.8% beta pinene, 0.3% ocimene, 0.2% beta cariophyllene besides other essential oil components in trace amounts. The chemical fingerprint details of CA have been described in our earlier publication (Ramachandran et al. 2015a).

2.3 Cytotoxicity assay

Breast carcinoma (MCF-7), cancer associated fibroblasts (CAF-05), normal fibroblasts (CCL-110) and HPAd adipocytes were treated with increasing concentrations of CA in DMEM for 72 h in 96 well plates. MTT [3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay performed with the Cell Proliferation Kit I (Roche Biochemicals, IN) was used to analyze cytotoxicity of CA. The experiments were repeated four times with three replications for each treatment and the IC_{50}, IC_{75} as well as IC_{90} values were calculated from absorbance readings (Ramachandran et al. 2015b; 2017).

2.4 ATP inhibition

Breast cancer cells (MCF-7), CAF-05, CCL-110 and HPAd cells ($5x10^3$/100 l medium/well) were plated in 96-well plates and incubated overnight at 37°C in a CO_2 incubator. Co-cultures were also grown by mixing MCF-7 cells with CAFs, normal fibroblasts or adipocytes in 1:1 ratio. On the next day, cells were treated with increasing concentrations of CA for 24 h in the CO_2 incubator. The plates were kept for 10 min at room temperature and 100 l of Cell Titer–Glo reagent (Promega Corporation,

Madison, WI) was added into the wells and mixed well for 10 times. The plates were shaken on an orbital shaker for 10 min and kept at room temperature for another 10 min for stabilization of luminescent signal and 100 l of sample was transferred to a fresh 96-well plate before the plates were read in the Veritas Luminometer. The ATP in each well was calculated considering the ATP level in the control (untreated) as 100%. The experiment was repeated four times and the average values plotted.

2.5 Lactate Inhibition

Breast cancer cells (MCF-7), cancer associated fibroblasts (CAF-05), normal fibroblasts (CCL-110) and HPAd adipocytes ($1x10^5$/ 2 ml/well) were plated in 24-well plates and incubated overnight at 37°C in a CO_2 incubator. Co-cultures were also grown by mixing MCF-7 cells with CAFs, normal fibroblasts or adipocytes in 1:1 ratio. After 24 h of plating, cells were treated with increasing concentrations of CA followed by incubation at 37°C for another 24 h in a CO_2 incubator. About 0.5 ml medium was collected from each well for estimation of lactate and 1 ml of 8% perchloric acid was added into the media. The mixture was vortexed well, kept at 4°C for 5 min. and centrifuged for 10 min at 1500 x g. About 25 l of the extract was combined with the 1.475 l of working solution containing nicotinamide adenine dinucleotide hydrate (NAD), glycine buffer and lactate dehydrogenase for 30 min at room temperature. The reaction mix (200 µl) was transferred to 96-well plate and the plate was read at 340 nm within 10 min. The relative amounts of lactate in the medium was calculated based on the lactate standard curve and plotted against drug concentrations.

2.6 Gene Expression Studies by RT-PCR Assay

Monocultures and co-cultures ($3 x10^6$/5 ml) were treated with increasing concentrations of CA for 72 h and the mRNA expression of genes associated with Warburg effect (MCT1, MCT4, HIF-1, Caveolin-1 and GLUT1) were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) (Ramachandran et al. 2015a; 2015b; 2017). The mRNA expression of a housekeeping gene, beta-actin, was used as a control. The gene expression levels were quantified using gel pictures by the UNSCAN-IT gel™ software (Silk Scientific, Inc., Orem, UT). The relative increase or decrease in mRNA level was calculated based on untreated sample and fold-level changes were plotted against CA concentrations.

2.7 Western blot analysis

Monocultures and co-cultures ($3 x10^6$/5 ml) were treated with various concentrations of CA for 72 h and total cellular protein was extracted using 0.5 ml of Invitrogen's protein extraction buffer (Invitrogen Corporation, Frederick, CA). The protein concentration was determined and 100 µg protein was separated on 10% SDS-PAGE. The separated protein was blotted on a nitrocellulose filter. The filters were hybridized with anti-human monoclonal/polyclonal antibodies specific for each Warburg effect- associated protein (MCT1, MCT4, HIF-1, Caveolin-1, and GLUT1 with β-actin control) in a western blot procedure and detected using the alkaline phosphatase color detection kit (Bio Rad Laboratories, Hercules, CA). The relative expression of proteins compared to untreated control samples were quantified using UNSCAN-IT gel™ software (Silk Scientific, Inc., Orem, UT). The relative increase or decrease in protein level was calculated based on untreated sample and fold-level changes were plotted against CA concentration.

2.8 Immunoperoxidase staining of monocultures and co-cultures with glycolysis markers

CAF-05, CCL-110, HPAd and MCF-7 cells were grown in chamber slides and treated with CA (0-5 g/ml) for 48 h. The monolayer cells on the chamber slides were digested with pepsin (1 mg/ml in Tris-HCl, pH 2.0) for 10 min at 37°C for unmasking of antigens. The slides were incubated in 3% hydrogen peroxide solution for 5 min, followed by washing and blocking with blocking solution. The slides were reacted with primary antibodies of glycolysis markers (Caveolin-1, MCT-1, MCT-4 , GLUT1 and HIF-1 alpha) overnight at 4°C, followed by three drops of detection reagent (secondary antibody) for 30 min at room temperature. The slides were stained according to the protocol described in the Immunohistochemistry Application Solutions kit (Cell Signaling technology, Danvers, MA).

2.9 Statistical Analysis

Mean and standard deviation estimates were calculated using Microsoft Excel software using data from three separate experiments. The dose-dependent trends in relative mRNA and protein expression were ascertained with samples treated with increasing CA concentrations. The relative mRNA and protein expression levels (fold change) at different CA concentrations were statistically analyzed by 1-way analysis of variance, and the treatments were compared with control treatment using Bonferroni's multiple comparison test (GraphPad Prism software, La Jolla, CA). The protein expression data of the CA-treated samples were statistically compared with the expression levels in untreated samples of CAF-05, CCL-110, HPAd, MCF-7 and co-cultures (*$p<0.05$, **$p<0.01$ and ***$p<0.001$).

3. Results

3.1 Cytotoxicity

The IC values of CA for CAF-05, CCL-110, HPAd and MCF-7 cell lines are given in Table 1. CA is highly cytotoxic to CAF-05 and MCF-7 cell lines at the IC_{90} level compared to HPAd and CCL-110 cell lines. The IC_{50}, IC_{75} and IC_{90} values for CA treatment of MCF-7 cells are 38.3, 45.3 and 48.3 µg/ml respectively. The IC_{50} value for HPAd cells on the other hand is 149.5 µg/ml and IC_{75} and IC_{90} values are > 200 µg/ml. For the CAF cell line, the IC_{50}, IC_{75} and IC_{90} values for CA are 24, 40.5 and 47.5 µg/ml, respectively. Therefore, both human pre-adipocytes and

normal fibroblasts are less susceptible to the direct cytotoxic effects of CA which demonstrates relative selective cytotoxicity against MCF-7 cancer cells and CAFs at relatively low doses.

3.2 ATP synthesis

The effect of CA on ATP synthesis in CCL-110 and MCF-7 mono-cultures and co-cultures is presented in Fig. 1. The effect of CA on ATP synthesis in CAF-05 and MCF-7 mono-cultures and co-cultures is presented in Fig. 2. When the CCL-110 cells are co-cultured with MCF-7, the inhibitory effect of CA is more pronounced in it than monocultures of MCF-7 and CCL-110 (Fig. 1). However, when the CAF-05 cells are in co-cultured with MCF-7, the rate of inhibition of ATP synthesis is highest in CAF-05 cell line followed by CAF-05+MCF-7 co-culture and MCF-7 monoculture in order.CA also inhibits ATP synthesis in HPAd pre-adipocytes (Fig. 3). The inhibitory effect of CA on ATP synthesis is more pronounced in HPAd, than in co-cultures with MCF-7 and MCF-7 monoculture. MCF-7 cells showed a slightly lower inhibition profile than co-culture at lower concentrations of CA (<50 μg/ml) but no difference at higher CA concentrations.

3.3 Lactate synthesis

CCL-110 showed a lower pre-treatment level of lactate synthesis than MCF-7 cells and their co-culture (Fig. 4a). CA treatment showed a dose-dependent inhibition of lactate synthesis and percentage of inhibition appeared to be higher in co-culture than monocultures of CCL-110 and MCF-7 (Fig. 4b).

Similar to CCL-110 cells, CAF-05 demonstrated lower lactate synthesis than MCF-7 cells and co-culture of CAF-05 + MCF-7 cells (Fig. 5a). CA treatment of monocultures of CAF-05 and MCF-7 and their co-culture inhibits lactate synthesis in a dose-dependent manner at CA concentrations <50 μg/ml. However, when the percentage of inhibition is calculated based on the untreated control, CA treatment showed an increased rate of lactate inhibition in the co-cultures compared to monocultures (Fig. 5b). Human pre-adipocytes (HPAd) also have lower level of lactate synthesis than MCF-7 cells and CA treatment inhibited the lactate synthesis in a dose-dependent manner (Fig. 6a). Similar to lactate synthesis in CAF-05 + MCF-7 and CCL-110 + MCF-7 co-cultures, the rate of inhibition of lactate synthesis in MCF-7 + HPAd co-culture by CA appeared to be slightly more than monocultures of MCF-7 and HPAd (Fig. 6b).

3.4 mRNA expression of genes associated with Warburg effect

The mRNA expression of genes associated with Warburg effect in CAF-05, MCF-7 cells and their co-culture treated with CA (0, 1 and 2 μg/ml) is presented in Fig.7. The untreated and treated cells in all cases demonstrate expression of MCT1, MCT4, HIF-1, Caveolin-1 and GLUT1 mRNA expression, almost at similar levels. The level of mRNA expression of MCT1, MCT4 and Caveolin-1 genes in MCF-7 cells were comparatively lower than that of CAF-05 and co-cultures (Fig.7)

The normal fibroblasts (CCL-110), MCF-7 and their co-culture also demonstrate mRNA expression of genes associated with Warburg effect (Fig.8). CA treatment (2 μg/ml) of the co-culture reduced the mRNA expression of MCT1 by 28% and Caveolin-1 by 34%. Also, CA treatment (2 μg/ml) inhibited Caveolin-1 mRNA expression of MCF-7 cells by 29%.Mono-cultures of human adipocytes (HPAd), breast cancer (MCF-7) cells and their co-culture demonstrate abundant mRNA for genes associated with Warburg effect (Fig. 9). However, MCF-7 cells have a significantly lower level of expression of MCT1, MCT4, HIF-1 and Caveolin-1 mRNAs compared to the co-culture (HPAd + MCF-7). The Caveolin-1 mRNA was down regulated by CA treatment of MCF-7 and co-cultures. MCF-7 and co-culture cells have a higher mRNA expression of GLUT1 gene compared to adipocytes.

3.5 Protein expression of genes associated with Warburg Effect

Western blot analysis of genes associated with Warburg effect in CAF-05, MCF-7 and co-culture is shown in Fig.10a and the quantification is given in Fig. 10b. Unlike mRNA expression, CAF-05 cells when cultured alone, failed to synthesize any MCT1, MCT4, HIF-1, Caveolin-1 and GLUT1 proteins. This translational inhibition of proteins associated with Warburg effect in CAF-05 cells was unexpected. However, this phenomenon is reproducible and thus requires further investigation. MCF-7 cells demonstrate abundant production of MCT1, MCT4, HIF-1, Caveolin-1 and GLUT1 proteins that was relatively unaffected by CA treatment. However, CAF-05 + MCF-7 co-culture treated with CA demonstrated decreased levels of proteins except HIF-1 and GLUT1. While treatment of co-culture with 5 g/ml of CA has reduced the synthesis of MCT1, MCT4, and Caveolin-1 proteins significantly; CA treatment at the 5 μg/ml dose has reduced the synthesis of MCT1 by 45%, MCT4 by 47%, and Caveolin-1 by 47%.

cultures were compared by 1-way analysis of variance with Bonferroni's multiple comparison test (GraphPad Prism software, La Jolla, CA. (*p<0.05, **p<0.01, ***p<0.001).

The normal fibroblast cell line CCL-110 showed undetectable levels of MCT1, MCT4, Caveolin-1 and GLUT1 proteins (Fig. 11a), even though these cells showed significant mRNA synthesis of these genes. Similar to CAF-05 cells, the translation of protein is inhibited in monoculture and warrants further investigation. However, unlike CAF-05 cells in monoculture, treatment of CCL110 + MCF-7 co-culture with low doses of CA has inhibited only the synthesis of HIF-1. CA at 5 μg/ml dose decreased the HIF-1 by 36% (Fig. 11b)

The protein expression pattern in HPAd adipocytes, MCF-7 cancer cells and co-cultures with and without CA treatment is given in Fig.12a and the quantification is presented in Fig. 12b. Similar to the CAF-05 and CCL-110 cells (although the house-keeping gene beta actin is expressed in all cases), adipocytes have no protein expression of glycolytic markers in monoculture, even though mRNAs were observed in the RT-PCR assay. MCF-7 cells as previously shown have abundant expression of these markers that could be modulated with higher doses (>10 µg/ml) of CA treatment (data not shown). CA treatment (5 µg/ml) of co-cultures with differentiated HPAd adipocytes + MCF-7 cells reduced protein expression of MCT1 by 55%, MCT4 by 36% and HIF-1alpha by 34% and GLUT1 by 25%.
cultures were compared by 1-way analysis of variance with Bonferroni's multiple comparison test (GraphPad Prism software, La Jolla, CA. (*p<0.05, **p<0.01, ***p<0.001).

3.6 Immunoperoxidase staining

Some of the results of immunoperoxdiase staining are presented in Fig. 13. CAF-05 cells possess very little expression of Caveolin-1 and MCT1 expression where as MCF-7 cells have abundant expression of these proteins. Treatment of co-cultures with CA (5 g/ml) for 48 h has reduced the expression of Caveolin-1 and MCT1 expression significantly. The results of immunoperoxidase staining of HPAd, MCF-7 and co-cultures showed results similar to western blots (data not shown).

4. Discussion

Although cancer cells are considered as the primary drivers of tumor development, it is now clear that other cells such as stromal cells (fibroblasts, adipocytes, endothelial cells, etc.) and also immune cells contribute to the tumorigenic microenvironment that enables cancer progression (Hanahan and Coussens 2012). For example, it has been well established that stromal fibroblasts infiltrate and are induced to differentiate into cancer associated fibroblasts (CAFs) (Ronnov-Jessen and Peterson 1993) and that they contribute to the energetics of tumor cells by fuelling them with energy sources like lactate, ketones, glutamine, fatty acids or cysteine (Bartling et al. 2008; Neiman et al. 2011;Martinez-Outschoorn 2014). Similarly, through lactate-induced extracellular acidification and secretion of inflammatory cytokines and matrix-degrading enzymes, CAFs enable metastasis and confer resistance to chemotherapeutic

Table 1: Cytotoxicity of supercritical CO_2 extract of mango ginger (CA) in cancer associated fibroblasts (CAF-05), normal fibroblasts (CCL-110), human adipocytes (HPAd) and breast cancer (MCF-7) cell lines

Cell line	IC_{50} (µg/ml)	IC_{75} (µg/ml)	IC_{90} (µg/ml)
CAF-05	24.0±2.1	40.5±2.5	47.5±0.7
CCL-110	36.7±0.6	49.0±1.4	>200
HPAd	149.5±4.9	>200	>200
MCF-7	38.3±1.2	45.3±1.1	48.3±2.1

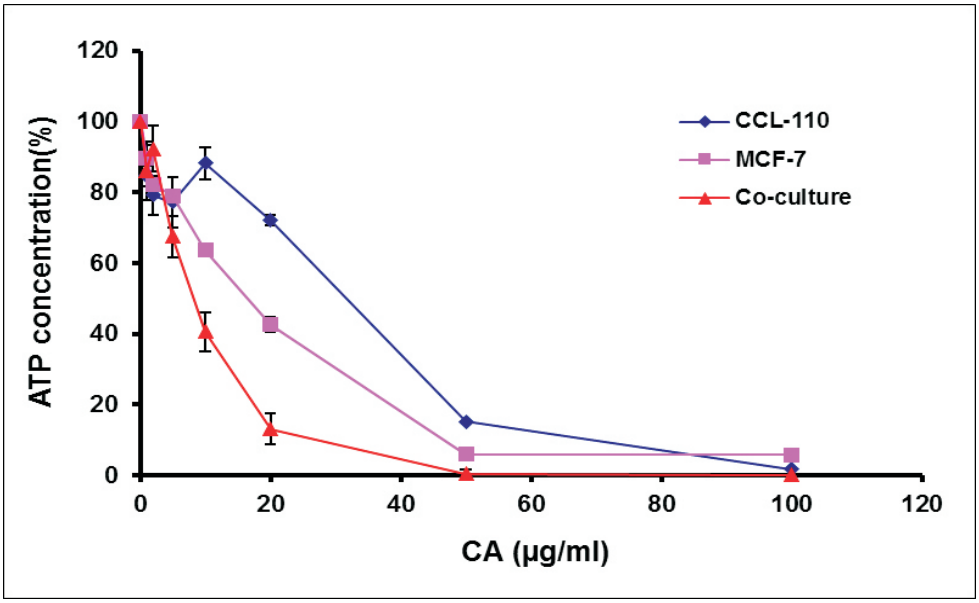

Figure 1. Inhibition of ATP synthesis by supercritical CO_2 extract of mango ginger (Curcuma amada Roxb.), CA in normal fibroblasts (CCL-110), breast cancer cells (MCF-7) and CCL-110 + MCF-7 co-cultures.

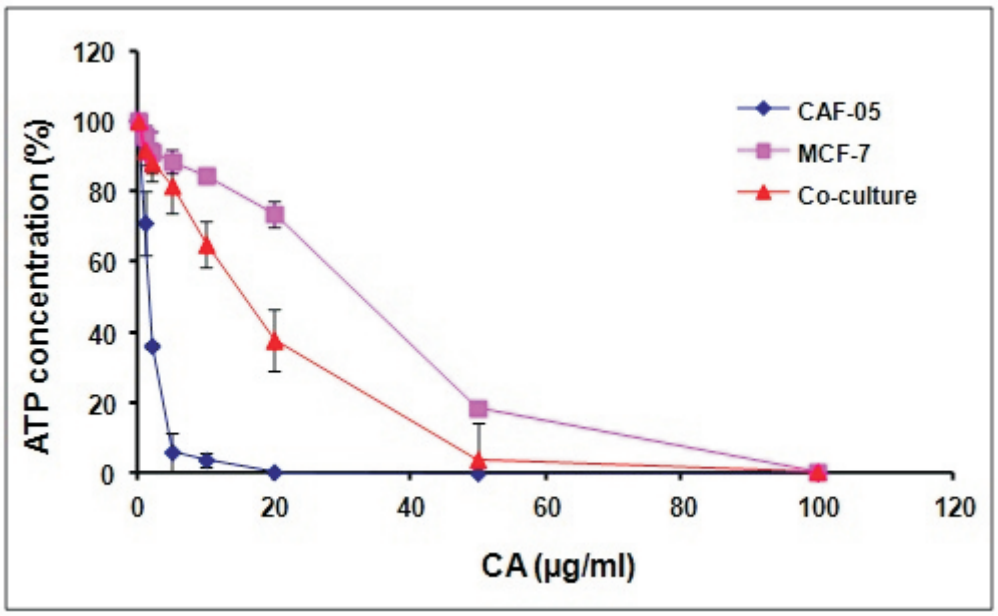

Figure 2. Inhibition of ATP synthesis by CA in cancer associated fibroblast (CAF-05), breast cancer cells (MCF-7) and CAF-05+MCF-7 co-cultures.

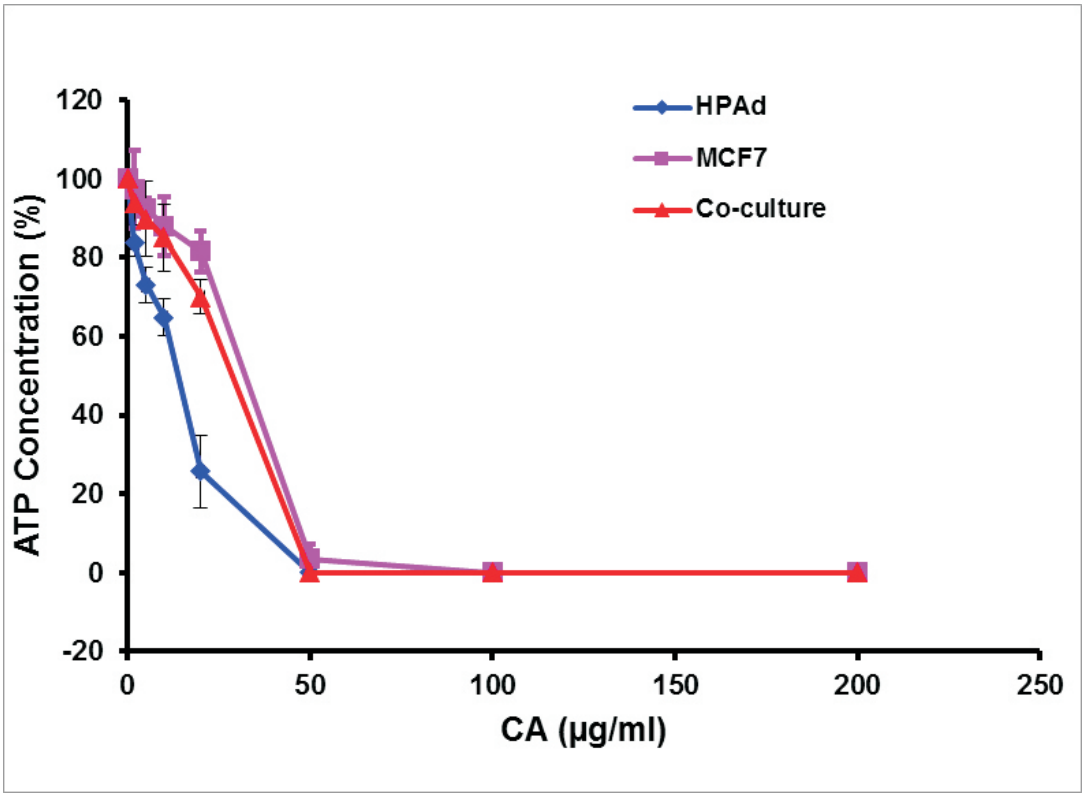

Figure 3. Inhibition of ATP synthesis by CA in breast adipocytes (HPAd), breast cancer cells and HPAd+MCF-7 co-cultures.

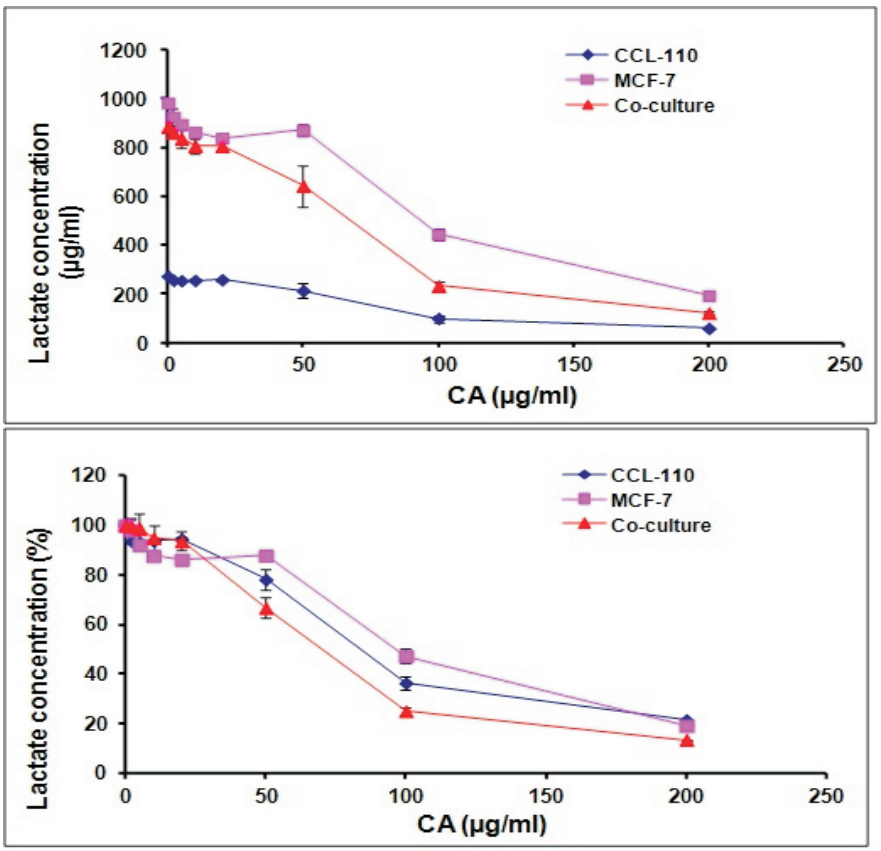

Figure 4. Inhibitory effect of CA on lactate synthesis in normal fibroblasts (CCL-110), breast cancer cells (MCF-7) and CCL-110+MCF-7 co-culture. (4a) Inhibition of total lactate in µg/ml concentration (4b) Inhibition of lactate as a percentage of untreated cells.

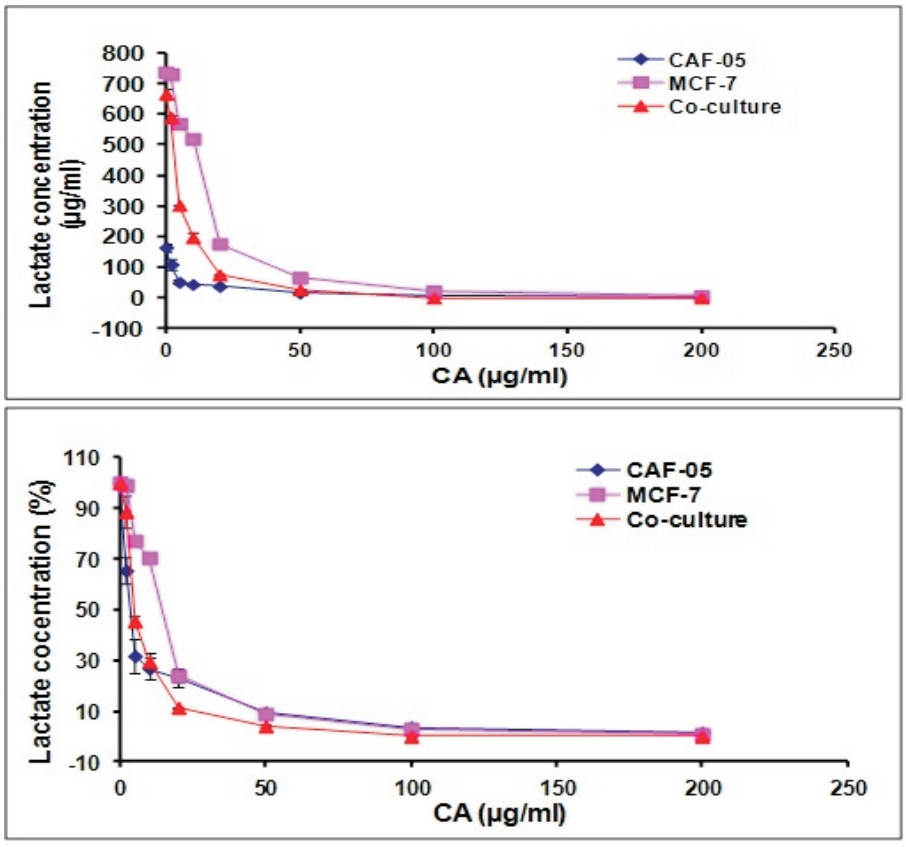

Figure 5. Inhibitory effect of CA on lactate synthesis in cancer associated fibroblasts (CAF-05), breast cancer cells (MCF-7) and CAF-05+MCF-7 co-cultures. (5a) Inhibition of total lactate in µg/ml concentration (5b) Inhibition of lactate as a percentage of untreated cells.

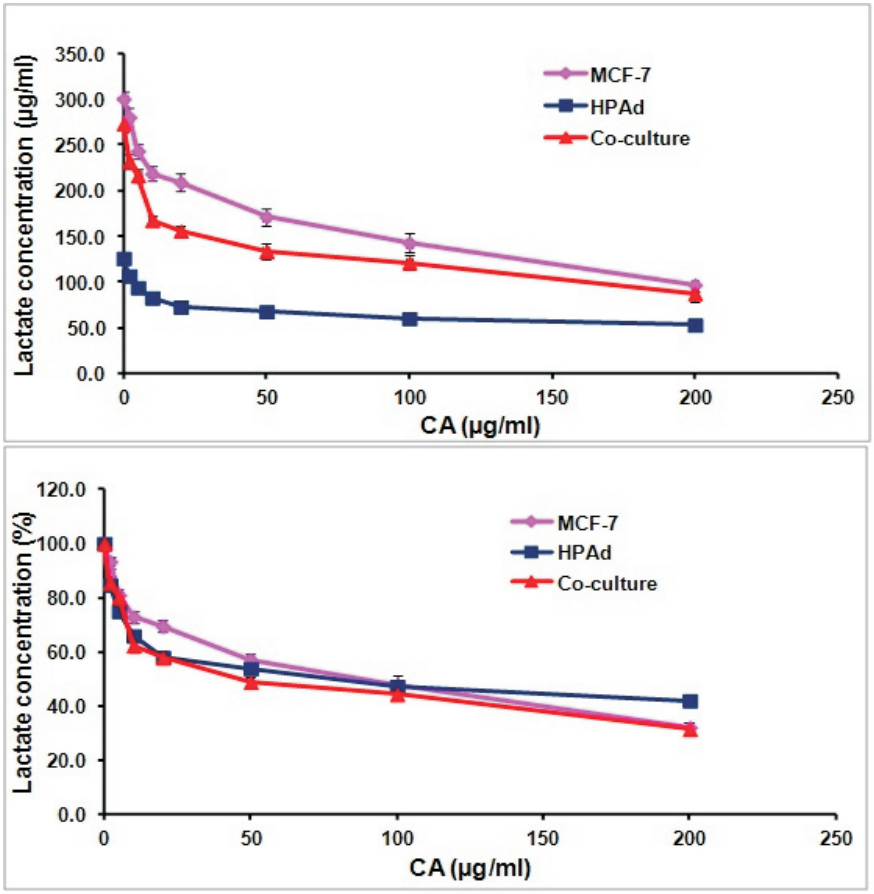

Figure 6. Inhibitory effect of CA on lactate synthesis in hµman adipocyte (HPAd), breast cancer (MCF-7) and HPAd+MCF-7 co-cultures. (6a) Inhibition of total lactate in µg/ml concentration; (6b) Inhibition of lactate as a percentage of untreated cells.

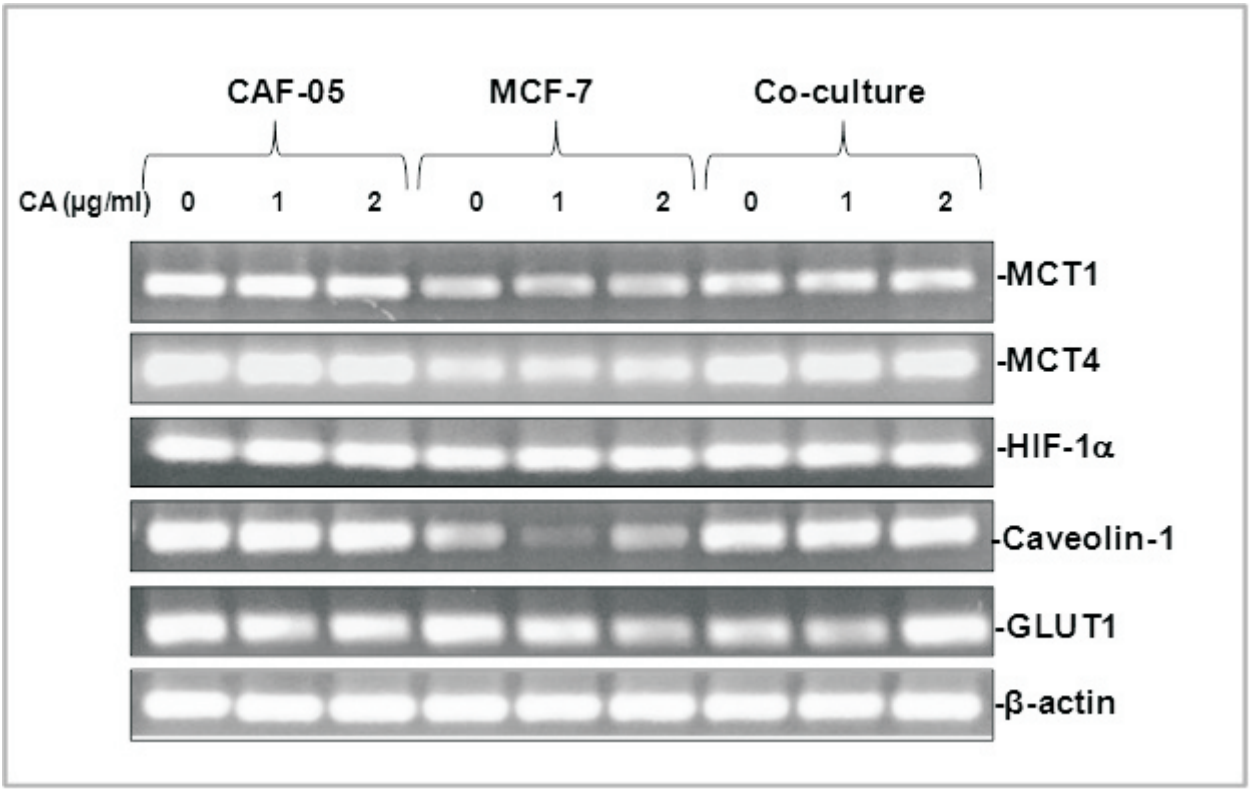

Figure 7. RT-PCR assay of genes associated with Warburg effect (MCT1, MCT4, HIF-1a, Caveolin-1, GLUT1) along with house keeping gene (beta-actin) in CAF-05, MCF-7 and co-cultures treated with CA (0, 1 and 2 µg/ml).

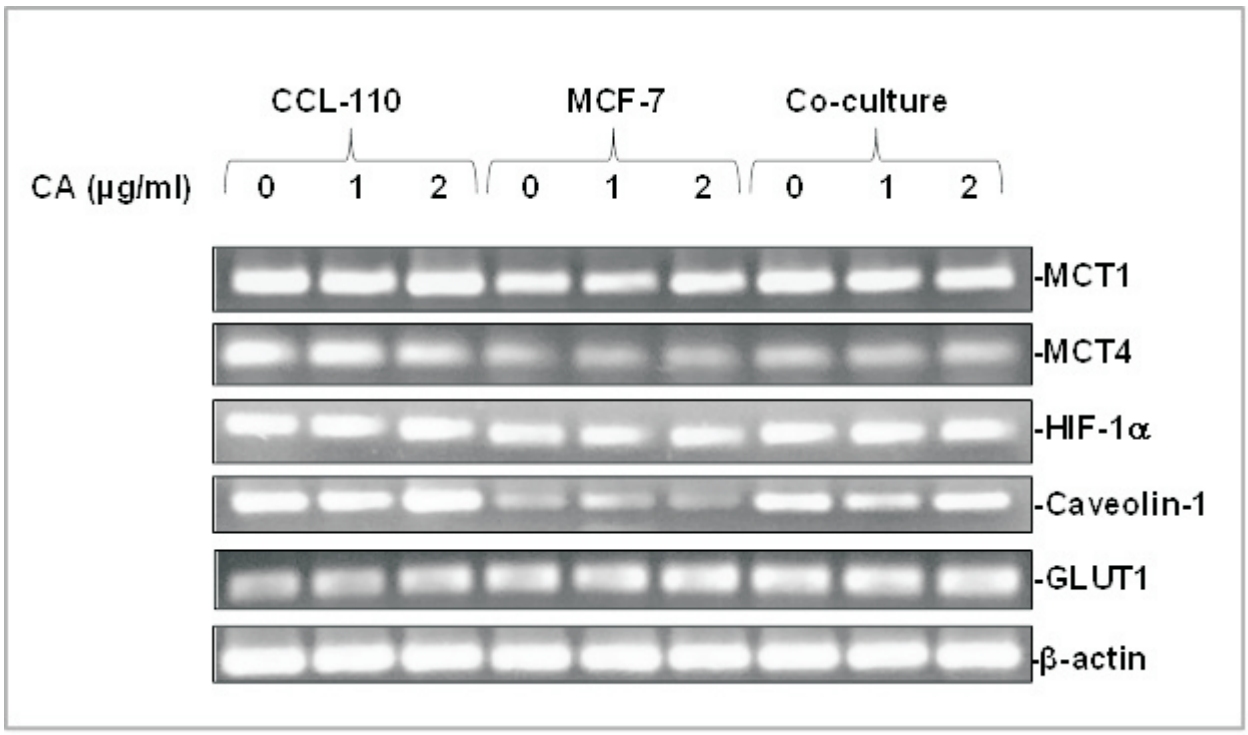

Figure 8. RT-PCR assay of genes associated with Warburg effect (MCT1, MCT4, HIF-1, Caveolin-1, GLUT1) along with house keeping gene (beta-actin) in CCL-110, MCF-7 and co-cultures treated with CA (0, 1 and 2 µg/ml).

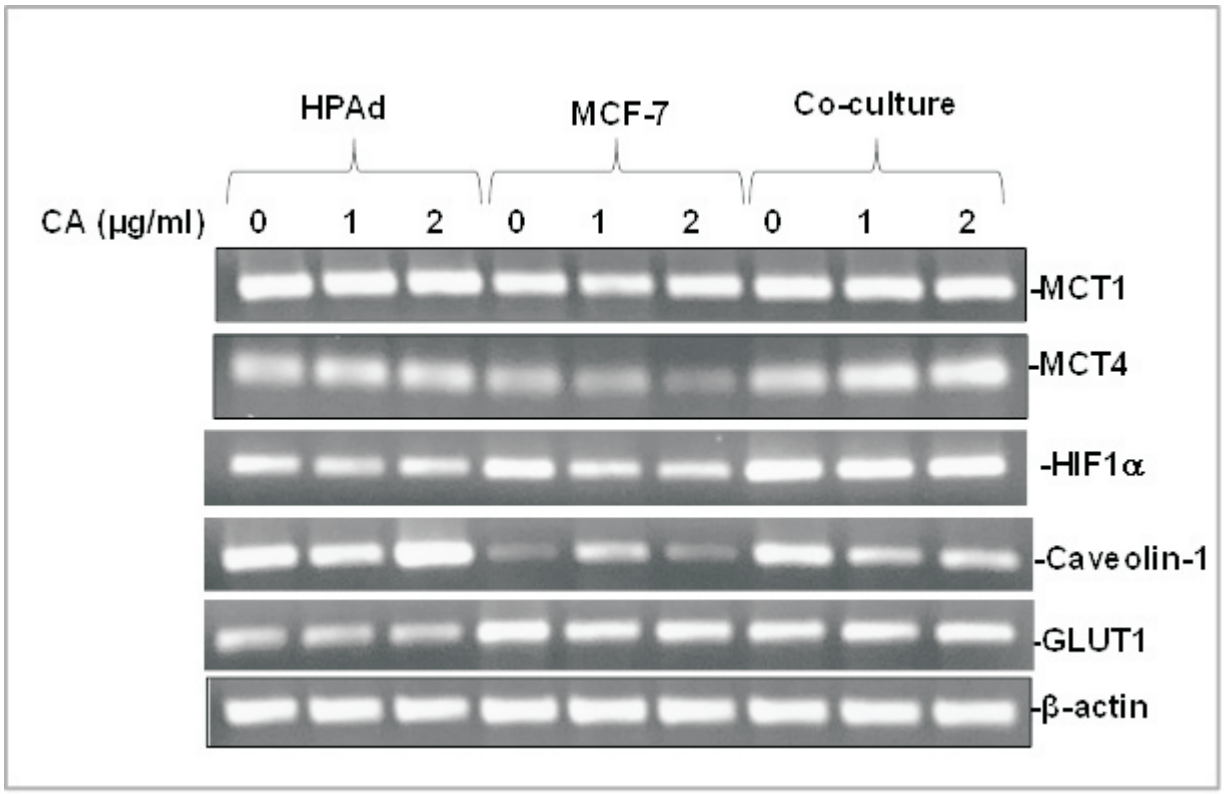

Figure 9. RT-PCR assay of genes associated with Warburg effect (MCT1, MCT4, HIF-1, Caveolin-1, GLUT1) along with house keeping gene (beta-actin) in HPAd, MCF-7 and co-cultures treated with CA (0, 1 and 2 µg/ml).

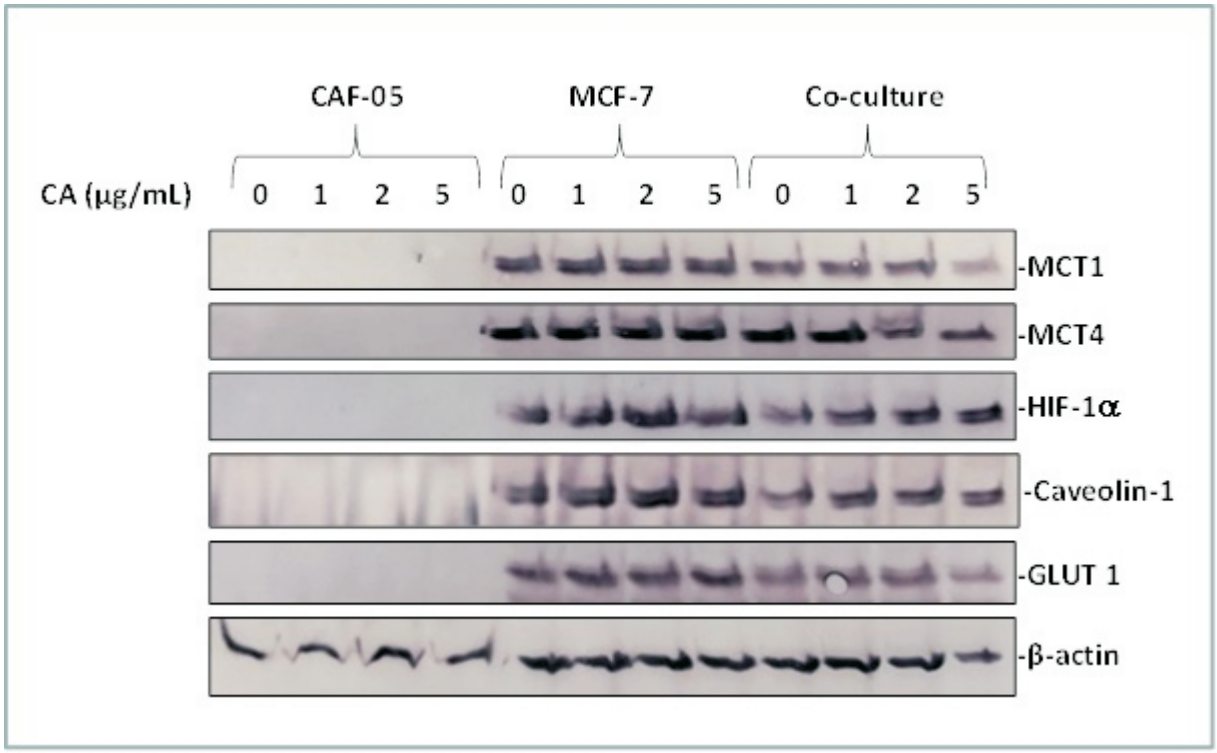

Figure 10a. Western blot analysis of expression of proteins associated with Warburg effect (MCT1, MCT4, HIF-1, Caveolin-1, GLUT1) along with house keeping gene (beta-actin) in CAF-05, MCF-7 and co-cultures treated with CA (0-5 µg/ml).

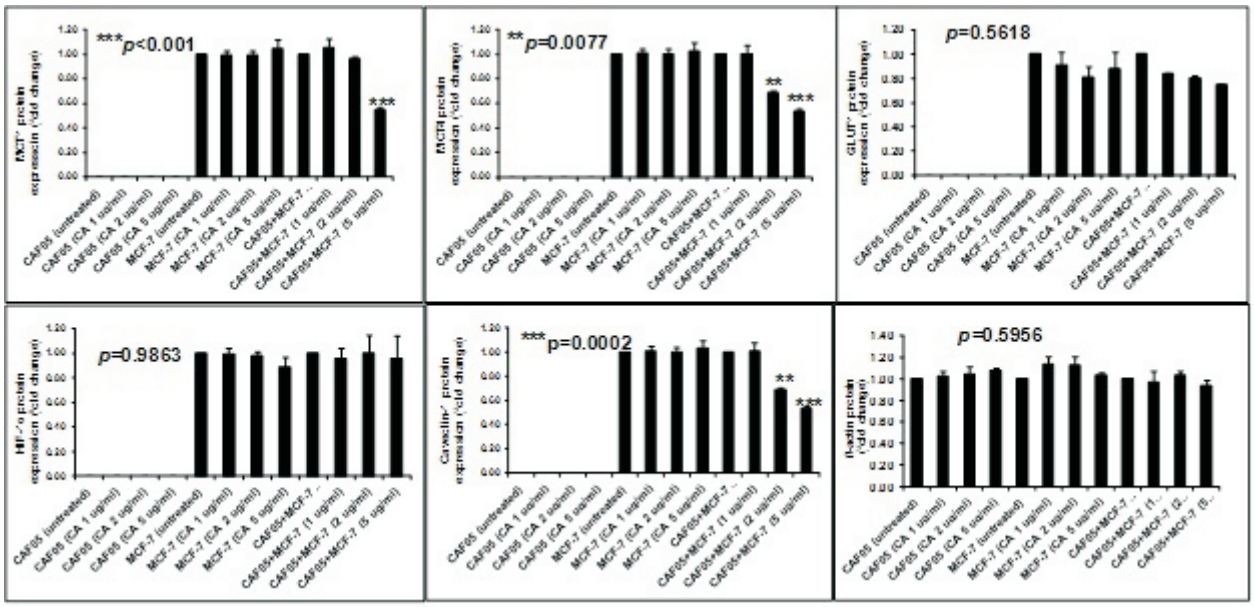

Figure 10b. Quantification of expression of proteins (western blots) associated with Warburg effect (MCT1, MCT4, HIF-1, Caveolin-1, and GLUT1) along with house keeping gene (beta-actin) in monocultures of CAF-05 and MCF-7 as well as their co-culture. The significant difference between CA treatments within monocultures and co-cultures were compared by 1-way analysis of variance with Bonferroni's multiple comparison test (GraphPad Prism software, La Jolla, CA. (*p<0.05, **p<0.01, ***p<0.001).

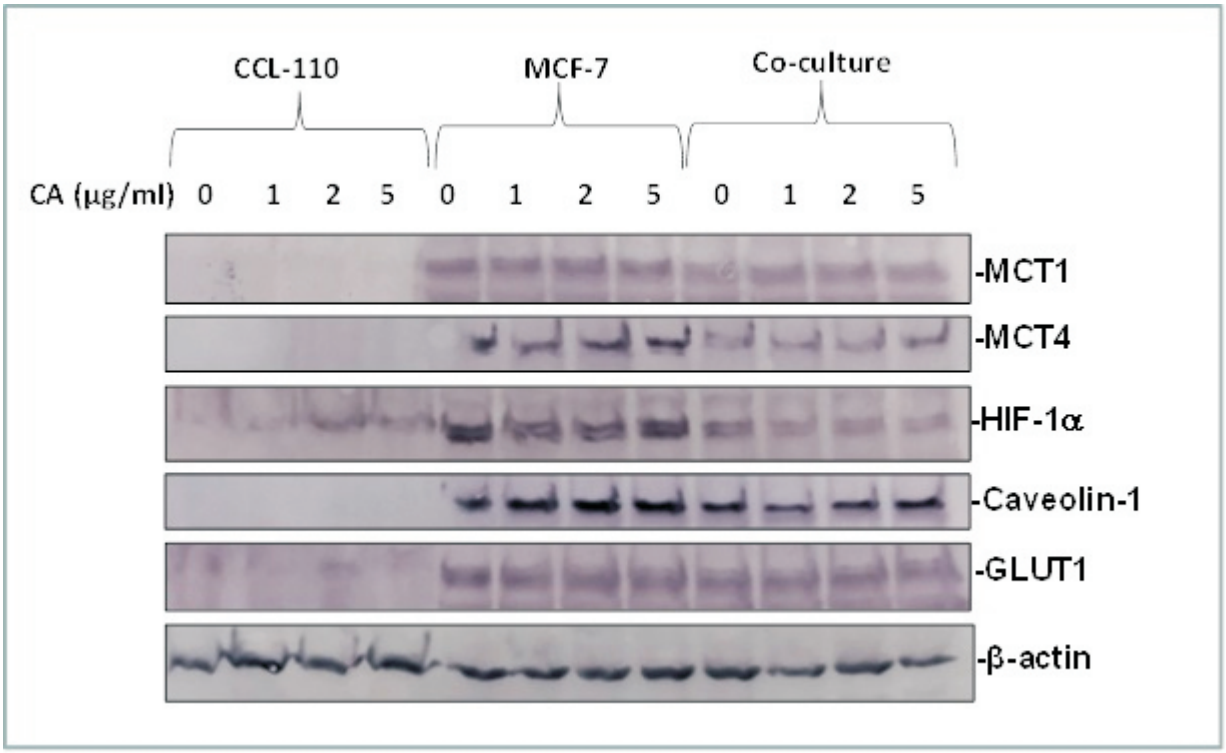

Figure 11a. Western blot analysis of expression of proteins associated with Warburg effect (MCT1, MCT4, HIF-1, Caveolin-1, GLUT1) along with house Fig. 11a. Western blot analysis of expression of proteins associated with Warburg effect (MCT1, MCT4,

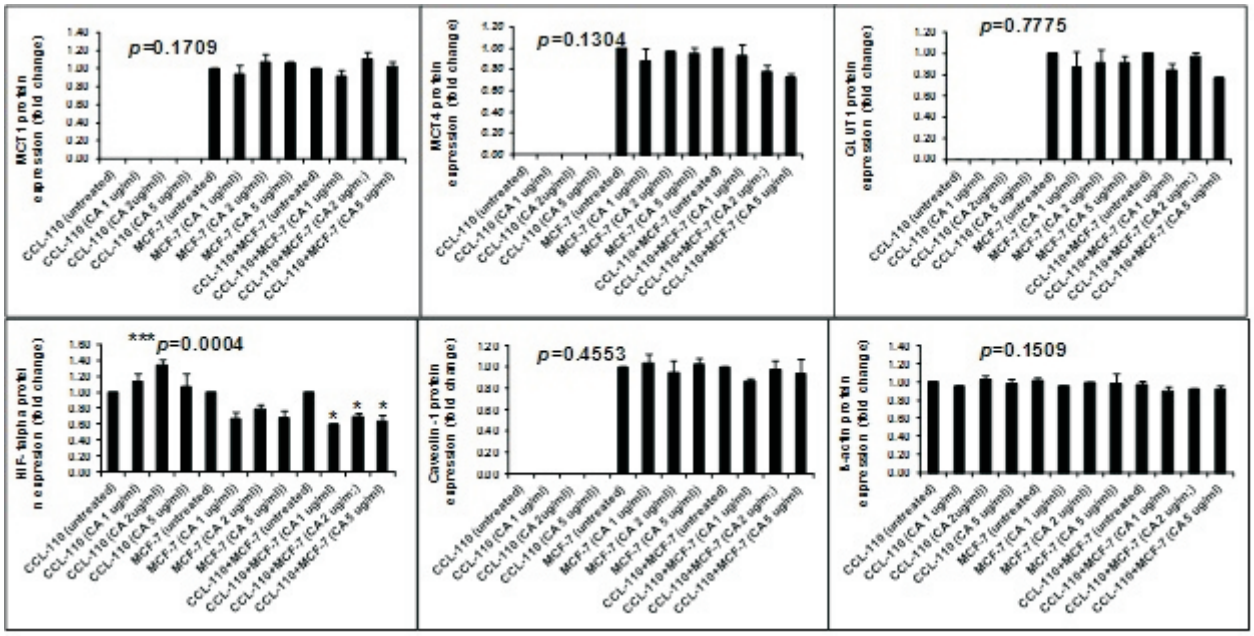

Figure 11b. Quantification of expression of proteins (western blots) associated with Warburg effect (MCT1, MCT4, HIF-1alphal, Caveolin-1, and GLUT1) along with house keeping gene (beta-actin) in monocultures of CCL-110 and MCF-7 as well as their co-culture. The significant difference between CA treatments within monocultures and co-cultures were compared by 1-way analysis of variance with Bonferroni's multiple comparison test (GraphPad Prism software, La Jolla, CA. (*p<0.05, **p<0.01, ***p<0.001).

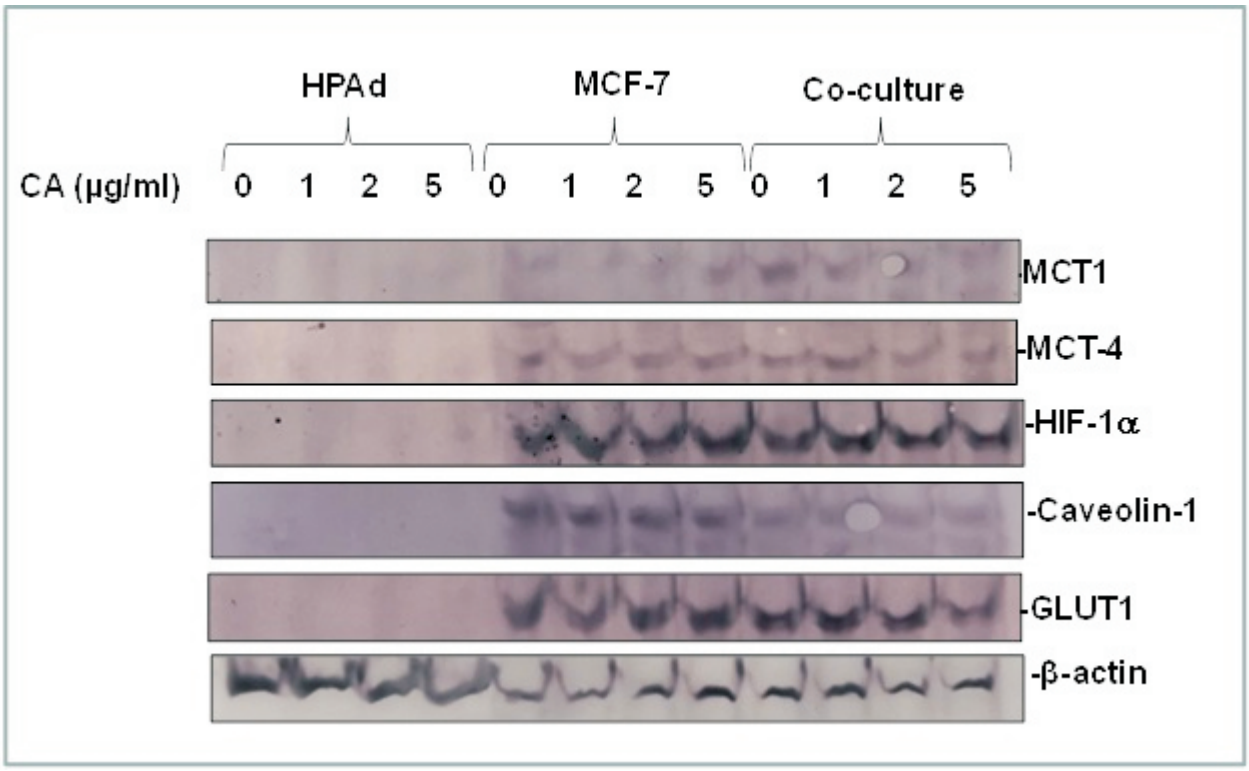

Figure12a. Western blot analysis of expression of proteins associated with Warburg effect (MCT1, MCT4, HIF-1, Caveolin-1 and GLUT1) along with house keeping gene (beta-actin) in HPAd, MCF-7 and co-cultures treated with CA (0-5 µg/ml).

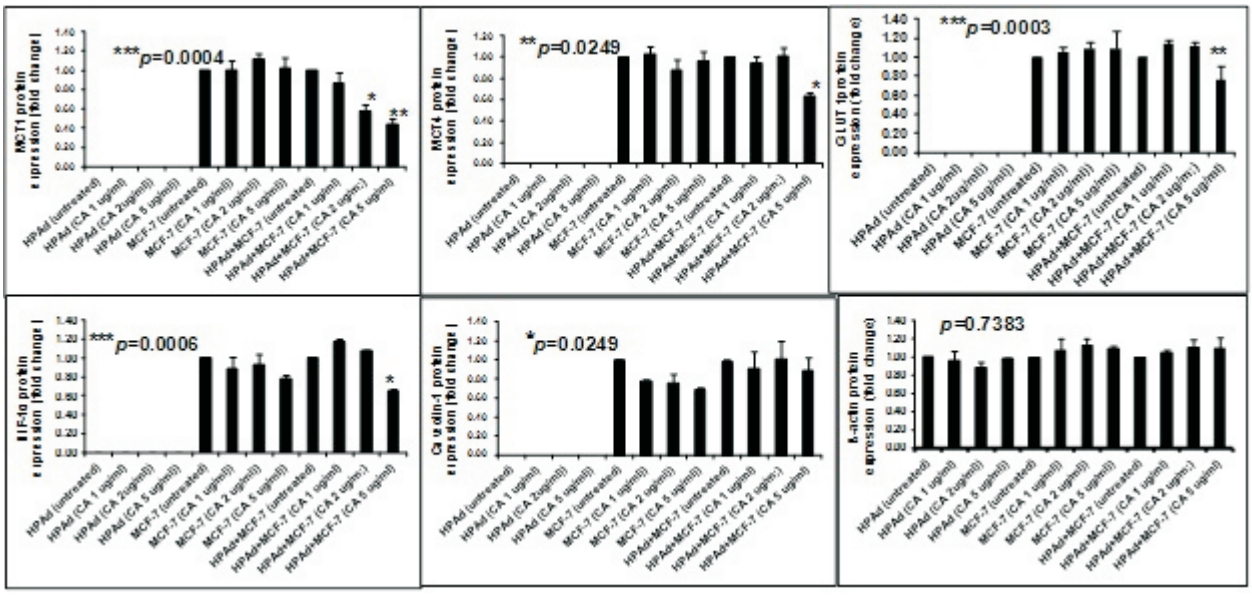

Figure 12b. Quantification of expression of proteins (western blots) associated with Warburg effect (MCT1, MCT4, HIF-1alphal, Caveolin-1, and GLUT1) along with house keeping gene (beta-actin) in monocultures of HPAd and MCF-7 as well as their co-culture. The significant difference between CA treatments within monocultures and co-cultures were compared by 1-way analysis of variance with Bonferroni's multiple comparison test (GraphPad Prism software, La Jolla, CA. (*p<0.05, **p<0.01, ***p<0.001).

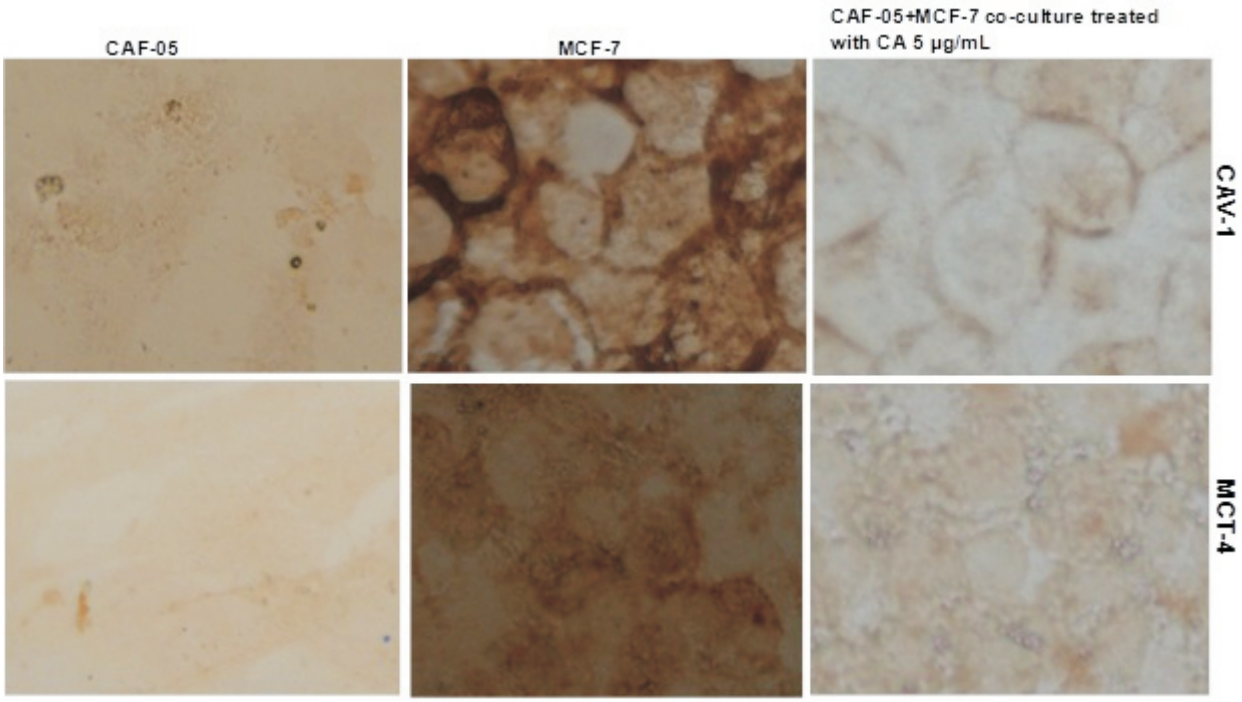

Figure 13. Immunoperoxidase staining of monocultures of CAF-05, MCF-7 and CA (5 µg/ml) treated co-cultures of CAF-05+MCF-7 with Caveolin-1 and MCT4 antibodies. Note the decrease in the expression of Caveolin-1 and MCT4 expression in co-cultures treated with CA.

drugs (Martinez-Outschoorn et al. 2011; Bartling et al. 2008; Laberge et al. 2012). In addition to CAFs, CAAs also contribute to the cancer growth and progression by providing energy sources (Diedrich et al. 2015; Balaban et al. 2017; Gupta et al. 2017). Therefore, more effective cancer treatment should also entail targeting of the tumor microenvironment including stromal cells such as cancer associated fibroblasts and adipocytes that fuel the cancer cells.

In the present investigation, we have shown that CA-treated CAF-05 and MCF-7 cells have lower IC values than CCL-110 fibroblasts and HPAd adipocytes. In our earlier investigations with CA, we have also reported that CA is cytotoxic to an array of cancer cells such as human rhabdomyosarcoma (both alveolar and embryonal), glioblastoma and leukemic cell lines while sparing cytotoxicity of non-malignant cell lines (Ramachandran et al. 2015a; 2015b; 2017). This preferential cytotoxicity of CA towards breast cancer and CAF cell lines would be an added attribute of this agent because simultaneous inhibition of cancer cells and CAFs would lead to a more effective inhibition of tumor growth and progression (Gupta et al 2017; Balaban et al. 2017 and Wen et al. 2017).

ATP analysis indicates that CA treatment has an inhibitory effect on ATP synthesis in monocultures of all four cell lines studied. However, the strongest inhibitory effects were observed with CA treatment in CAF-05 monoculture, MCF-7+CCL-110 co-culture and MCF-7+CAF-05 co-culture. Several investigators have reported that chemotherapy would be more effective by the addition of drugs that target elements of the TME in addition to cancer cells (Zhou et al. 2015; Luo et al. 2015; Slany et al. 2015). Consequently, it has been suggested that co-culture methods would be more useful for investigating synergy between tumor cells and stromal cells compared to individual monocultures in order to identify more effective therapeutic strategies (Tchou and Conego-Garcia 2012). In the present investigation increased ATP inhibitory effect of CA on co-cultures relative to MCF-7 monoculture has been demonstrated.

The analysis of lactate concentration levels in untreated and CA treated monocultures and co-cultures of cancer cells with normal fibroblasts, CAFs or adipocytes showed that cancer cells and co-cultures have higher lactate synthesis than monocultures of CAF-05, CCL-110 or HPAd cells and that the inhibition of lactate synthesis by CA occurred in a dose-dependent manner. The level of lactate synthesis by untreated monocultures of CAF-05, CCL-110 and HPAd is lower than MCF-7 and co-cultures; however, the lactate levels were further reduced by CA treatment. The levels of lactate synthesis in untreated MCF-7 cells and each co-culture are comparable; however, the inhibition of lactate synthesis induced by CA treatment is uniformly better in MCF-7 co-cultures than MCF-7 monoculture. While inhibition of lactate synthesis in cancer cells alone is a therapeutic target, inhibition of lactate synthesis in normal stromal cells and cancer-associated stromal cell co-cultures supports the role of the reverse Warburg effect by CA treatment (Teicher et al. 2012; Lisanti et al. 2013; Morandi and Charge 2014). These observations clearly support the concept that the biological behavior of cancer if not its initiation is very much dependent upon its stromal microenvironment. For example, it has been reported that ROS (reactive oxygen species) production drives the onset of CAF phenotype resulting in an amplification of ROS production, creating an

environment of oxidative stress. This has important functional consequences as ROS production in CAFs induces a shift towards aerobic glycolysis initiating the "reverse Warburg effect" (Pavides et al. 2009; Witkiewicz et al. 2012).

Each cell line in monoculture and the MCF-7/stromal cell co-culture we investigated, demonstrated mRNA expression of glycolysis-associated biomarkers; MCT1, MCT4, HIF-1 and GLUT1. Caveolin-1 mRNA is also expressed in these cells. However, monocultures of CAF-05, CCL-110 and HPAd did not possess any protein expression of these biomarkers. This phenomenon is reproducible and also independently observed in three different stromal cell lines in monoculture. While the explanation requires further evaluation, it is hypothesized that the higher levels of oxidative stress that is induced in the cancer microenvironment leads to adaptive redox changes of stromal cells that in turn induces synthesis of these glycolysis-associated proteins that would otherwise not occur in the absence of oxidative stress. Protein expression of glycolysis markers (MCT1, MCT4, HIF-1alpha and GLUT1) in CAF-05+MCF-7 and HPAd+MCF-7 co-cultures and their inhibition with CA (5 µg/ml) is quite apparent. HIF-1α controls several genes involved in glucose and lactate transport, such as GLUT1, MCT1 and MCT4. In addition HIF-1α signaling also affects pH stabilization and angiogenesis thereby affecting the tumorogeneis and metastasis (Eckert et al. 2016). It has been reported that inhibition of lactate transport by inhibition of MCT1 and MCT4 will inhibit glycolytic pathway and tumor growth. Such a strategy will affect the metabolic symbiosis between stromal cells (CAFs and CAA's) and cancer cells (Liu et al. 2016).

In the present research, CA (5 g/ml) also downregulated the protein expression of glycolysis markers MCT1, MCT4, HIF-1α and GLUT1 in HPAd+MCF-7 co-culture. It is hypothesized that reprogramming of the lipid metabolism in the tumor microenvironment via upregulation of biosynthesis and secretion of adipokines, growth factors, proteases, fatty acids and other factors may occur in the TME (Gupta et al. 2017, Romero et al.2015). In the past few years, adipocytes have emerged as another key contributor to the promotion of tumor proliferation and migration through a number of mechanisms such as glycolysis, Akt activation, IGF-1 amplification, autophagy induction and increase in the levels of fatty acid-binding proteins (FABP) that are now becoming better understood (Nieman et al. 2011; Gupta et al.2017).

In previous research that compliments the findings in this study, we showed that CA at higher concentrations above 10 g/ml inhibit ATP and lactate synthesis and has synergistic effects with synthetic glycolysis inhibitors like 2-deoxy-D-glucose and sodium oxamate in U-87MG glioblastoma cells (Ramachandran et al. 2019). Through the broadening of our understanding of the symbiosis of cancer and the stromal environments new therapeutic targets are emerging based on the inhibition of the Warburg effect in cancer cells and reverse Warburg effect in CAFs and CAAs. In this way compounds such as CA may potentially offer a more robust therapeutic approach to human malignancies.

5. Conflict of Interest

The authors declare the following conflict of interest with respect to the research, authorship, and/or publication of this article. Dr. Steven J. Melnick is the founder of Dharma Biomedical LLC, which is an evidence-based ethnobotanical and evochemical drug discovery and nutraceutical company operating on a for-profit basis. Dr. Karl-Werner Quirin is the Chief Executive Officer of Flavex Naturextrakte GmbH, Rehlingen, Germany, a company producing specialty botanical extracts for cosmetics and food supplements on the basis of supercritical CO_2 extraction. Dr. Cheppail Ramachandran and Ms. Ashley Juan are employees of Dharma Biomedical LLC.

6. Ethical Approval

This in vitro investigation did not involve any human subjects or live animals. Therefore, Institutional Review Board (IRB) and Institutional Animal Care and Use Committee (IACUC) approvals were not applicable.

References

[1]. Balaban S, Shearer RF, Lee LS, van Geldermalsen M, Schreuder M, Shtein HC et al. Adipocyte lipolysis links obesity to breast cancer growth: adipocyte-derived fatty acids drive breast cancer cell proliferation and migration. Cancer Metab. 2017 Jan 13;5:1. doi: 10.1186/s40170-016-0163-7.

[2]. Bartling B, Hofmann HS, Silber RE, Simm A. Differential impact of fibroblasts on the efficient cell death of lung cancer cells induced by paclitaxel and cisplatin. Cancer Biol. Ther. 2008, 7: 1250-1261.

[3]. Bissel MJ, Hines WC. Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. Nat. Med. 2011, 17: 320-329.

[4]. Bonnans C, Chou J, Werb Z. Remodeling the extracellular matrix in development and disease. Mol. Cell Biol. 2014, 14: 786-801.

[5]. Blucher C, Stadler SC. Obesity and breast cancer: current insights on the role of fatty acids and lipid metabolism in promoting breast cancer growth and progression. Front. Endocrinol. 2015, 8: 293-299.

[6]. Costa A, Scholer-Dahirel A, Mechta-Grigoriou F. The role of reactive oxygen species and metabolism on cancer cells and microenvironment. Semin. Cancer Biol. 2014, 25: 23-32.

[7]. Diedrich J, Gusky HC, Podgorski I. Adipose tissue dysfunction and its effects on tumor metabolism. Horm. Mol. Biol. Clin. Investig. 2015, 21: 17-41.

[8]. DiGirolamo M, Newby FD, Lovejoy J. Lactate production in adipose tissue: a regulated function with extra-adipose implications. FASEB J. 1992, 6: 2405-2412.

[9]. Dirat B, Bochet L, Dabek M, Daviaud D, Dauvillier S, Majed B, et al. Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion. Cancer Res. 2011, 7: 2455-2465.

[10]. Eckert AW Wickenhauser C, Salins PC, Kappler M,

Bukur J, Seliger B. Clinical relevance of the tumor microenvironment and immune escape of oral squamous cell carcinoma. J Transl. Med. 2016, 14: 85.doi: 10.1186/s12967-016-0828-6.

[11]. Gentric G, Mieulet V, Mechta-Grigoriou F. Heterogeneity in cancer metabolism: New concepts in an old field. Antioxidants and Redox Signaling 2017, 26: 462-485.

[12]. Gupta S, Roy A, Dwarakanath BS. Metabolic cooperation and competition in the tumor microenvironment: Implications for therapy Front. Oncol. 2017, 7: 68, doi: 10.3389/fonc.2017,00068.

[13]. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011, 144: 646-674.

[14]. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor environment. Cancer Cell 2012, 21: 309-322.

[15]. Hoy AJ, Balaban S, Saunders DN. Adipocyte-tumor cell metabolic crosstalk in breast cancer. Trends Mol. Med.2017, 23: 381-392.

[16]. Jansson PA, Larsson A, Smith U, Lonnroth P. Lactate release from the subcutaneous tissue in lean and obsese men. J. Clin. Invest. 1994, 93: 240-246.

[17]. Karangiannis GS, Poutahidis T, Erdmann SE, Kirsch R, Riddell RH, Diamandis EP. Cancer-associated fibroblasts drive the progression of metastasis through both paracrine and mechanical pressure on cancer tissue. Mol. Cancer Res. 2012, 10: 1403-1418.

[18]. Laberge RM, Awad P, Campisi J, Desprez PY. Epithelial-mesenchymal transition induced by senescent fibroblasts. Cancer Microenviron. 2012, 5: 39-4.

[19]. Liu M, Quek LE, Sultani G T, Turner N. Epithelial-mesenchyma transition induction is associated with augmented glucose uptake and lactate production in pancreatic ductal adenocarinoma. Cancer Metab. 2016, 4:19.

[20]. Luo H, Tu G, Liu Z, Liu M. Cancer-associated fibroblasts: a multifaceted driver of breast cancer progression. Cancer Lett. 2015, 36: 155-163.

[21]. Lisanti MP, Marinez-Outschoom UE, Sotgia F. Oncogenes induce the cancer-associated fibroblast phenotype: metabolic symbiosis and fibroblast addiction are new therapeutic targets for drug discovery. Cell Cycle 2013, 12: 2723-2732.

[22]. Martinez-Outschoorn UE, Lin Z, Ko YH, Goldberg AF, Flomenberg N, Wang C, et al. Understanding the metabolic basis of drug resistance: therapeutic induction of the Warburg effect kills cancer cells. Cell Cycle 2011, 10: 2521-2528.

[23]. Martinez-Outschoorn UE, Peiris-Pagés M, Pestell RG, Sotgia F, Lisanti MP. Cancer metabolism: a therapeutic perspective. Nat. Rev. Clin. Oncol. 2017; 14: 11-31.

[24]. Martinez-Outschoorn UE, Sotgia F, Lisanti MP. Tumor microenvironment and metabolic synergy in breast cancers: critical importance of mitochondrial fuels and function. Seminars in oncology 2014, 1: 195-216.

[25]. Morandi A, Charge P. Metabolic implication of tumor:stroma crosstalk in breast cancer. J. Mol. Med. 2014, 92: 117-126.

[26]. Nieman KM, Romero IL, Van Houten B, Lengyel E. Adipose tissue and adipocytes support tumorigenesis and metastasis. Biochim. Biophys acta 2013; 1: 189-206.

[27]. Nieman KM, Kenny HA, Penicka CV, Ladnyi A, Buell-Gutbrod R, Zillhardt MR. Adiopocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. Nat. Med. 2011, 17: 1498-1503.

[28]. Ohlund D, Elyada E, Tuveson D. Fibroblast heterogeneity in the cancer wound. J. Exp. Med. 2014, 211: 1503-1523.

[29]. Pavlides S, Whitaker-Menezes D, Castello-Cros R, Flomenberg N, Witkiewicz AK, Frank PG, et al. The reverse Warburg effect: aerobic glycolysis in cancer associated fibroblasts and the tumor stroma. Cell Cycle 2009, 8: 3984-4001.

[30]. Petersen C, Nielsen MD, Andersen ES, Basse AL, Isidor MS, Markussen LK et al. MCT1 and MCT4 expression and lactate flux activity increase during white and brown adipogenesis and impact adipocyte metabolism. Sci. Rep. 2017, 7:13101.

[31]. Ramachandran C, Lollett IV, Escalon E, Quirin K-W, Melnick SJ. Anticancer potential and mechanism of mango ginger (Curcuma amada Roxb.) supercritical CO2 extract in human glioblastoma cells. J. Evid. Based Complement. Altern. Med. 2015a, 20: 109-119.

[32]. Ramachandran C, Portalatin G, Quirin K-W, Escalon E, Khatib Z, Melnick SJ. Inhibition of AKT signaling by supercritical CO_2 extract of mango ginger (Curcuma amada Roxb.) in human glioblastoma cells. J. Complement. Integr. Med. 2015b, 12: 307-315.

[33]. Ramachandran C, Portalatin G, Prado AM, Quirin K-W, Escalon E, Melnick SJ. In vivo antitumor effect of supercritical CO2 extract of mango ginger (Curcuma amada Roxb.) in U-87MG human glioblastoma nude mice xenografts. J Evid. Based. Complement. Altern. Med. 2017, 22: 260-267.

[34]. Ramachandran C, Juan A, Qurin K-W, Khatib L, Schultz Y, Lampidis TL, Escalon E, Melnick SJ. Synergistic effect of supercritical CO2 extract of mango ginger (Curcuma amada Roxb.) with glycolytic inhibitors in human glioblastoma cell in vitro. Advances in Cancer Research and Therapy 2019, 3: 1-17

[35]. Rattigan YI, Patel BB, Ackerstaff E, Sukenick G, Koutcher JA, Glod JW et al. Lactate is a mediator of metabolic cooperation between stromal carcinoma associated fibroblasts and glycolytic tumor cells in the tumor microenvironment. Exp. Cell Res. 2012, 318: 326-335.

[36]. Romero IL, Mukherjee A, Kenny HA, Lichfield LM, Lengyel E. Molecular pathways: trafficking of metabolic resources in the tumor microenvironment. Clin. Cancer Res. 2015, 21: 680-686.

[37]. Ronnov-Jessen L, Petersen OW. Induction of alpha-smooth muscle actin by transforming growth factor-beta 1 in quiescent human breast gland fibroblasts. Implications for myofibroblast generation in breast neoplasia. Lab Invest. 1993, 68: 696-707.

[38]. Sabater D Arriarán S, Romero Mdel M, Agnelli S, Remesar X, Fernández-López JA et al. Cultured 3T3L1 adipocytes dispose of excess medium glucose as lactate under abundant oxygen availability. Sci. Rep. 2014, 3: 3663.

[39]. Slany A, Bileck A, Mugaku B, Gerner C. Targeting breast cancer-associated fibroblasts to improve

anti-cancer therapy. Breast 2015, 24: 532-538.
[40]. Tchou J, Conejo-Garcia J. Targeting the tumor stroma as a novel treatment strategy for breast cancer: shifting form the neoplastic cell-centric to a stroma-centric paradigm. Adv. Pharmacol. 2012, 65: 45-61.
[41]. Teicher BA, Linehan WM, Helman LJ. Targeting cancer metabolism. Clin. Cancer Res. 2012, 18: 5537-5545.
[42]. Wang YY, Lehuede C, Laurent V, Dirat B, Dauvillier S, Bochet L, et al. Adipose tissue and breast epithelial cells: a dangerous dynamic duo in breast cancer. Cancer Lett. 2012, 324: 142-151.
[43]. Warburg O. The origin of cancer cells. Science 1956, 123: 309-314.
[44]. Warburg O, Wind F. The metabolism of tumors in the body. J Gen Physiol. 1927, 8: 519-530.
[45]. Wen X, He X, Jiao F, Wang C, Sun Y, Ren X, Li Q. Fibroblast activation Protein-α-positive fibroblasts promote gastric cancer progression and resistance to immune checkpoint blockade. Oncol. Res. 2017, 25:629-640
[46]. Witkiewicz AK, Whitaker-Menezes D, Dasgupta A, Philip NJ, Lin Z,Gandara R, et al. Using the "reverse Warburg effect" to identify high-risk breast cancer parents: stromal MCT4
[47]. predicts proor clinical outcome in triple-negative breast cancer. Cell Cycle 2012, 11; 1108-1117.
[48]. Zhou L, Yang K, Andi T, Wickett RR, Zhang Y. Perspective of targeting cancer-associated fibroblasts in melanoma. J. Cancer 2015, 6: 717-716