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RESEARCH ARTICLE

Synergistic Effect of Supercritical CO_2 Extract of Mango Ginger (*Curcuma amada*-Roxb.) With Glycolytic Inhibitors in Human Glioblastoma Cells *In Vitro*

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ABSTRACT

Background: Cancer cells, unlike normal cells, principally use aerobic glycolysis with reduced mitochondrial oxidative phosphorylation for glucose metabolism, a phenomenon referred to as the Warburg effect. Glioblastoma, one of the most aggressive, lethal and incurable human tumors with a survival rate of 12-15 months in patients undergoing standard of care treatment involving surgery, chemotherapy and radiation therapy, has been shown to have a preferential metabolism of glucose through aerobic glycolysis. The cytotoxic effects of a previously described supercritical CO_2 extract of mango ginger (*Curcuma amada*-CA) with two glycolytic inhibitors [2-deoxy-D-glucose (2-DG) and sodium oxamate (SO)] was investigated in the U-87MG human glioblastoma cell line.

Methods: Cytotoxicity assay was performed with increasing concentrations of CA, 2-DG and SO as single agents and in combinations in U-87MG glioblastoma cells by the MTT assay. The cytotoxicity data was analyzed using CompuSyn software to determine the synergism/additive effect/antagonism between drugs. The effect of CA and glycolytic inhibitors on ATP and lactate synthesis was analyzed to establish the inhibitory effects of individual drugs as well as their combinations on glycolysis pathway. The modulatory effect of CA, 2-DG and SO as single agents or combinations on mRNA and protein expression of apoptotic and metastasis genes were also analyzed by RT-PCR and western blot hybridization, respectively.

Results: The hexokinase inhibitor 2-DG and the lactate dehydrogenase-A inhibitor SO, both inhibiting the glycolysis pathway, showed synergistic cytotoxic effects with CA in the glioblastoma cell line with combination index values of <1 in the CompuSyn analysis. CA inhibits cellular ATP synthesis in a dose-dependent manner and it has better inhibition profile than 2-DG and SO. CA inhibits cellular lactate synthesis significantly better than 2-DG and SO at low concentrations, and CA+2-DG combination appears to be better than single agents at low doses for lactate inhibition in glioblastoma cells. Gene expression analysis by RT-PCR and western blot hybridization showed that CA, 2-DG and SO as well as their combinations up regulate the ratio of Bax/Bcl-2, p21, TIMP1 and caspase-3 expression and down regulate mutant p53 and MMP2 expression that may increase apoptosis and inhibit cell proliferation as well as metastasis of tumor cells.

Conclusion: The combination of CA with glycolytic inhibitors like 2-DG and SO is beneficial for inhibition of growth, proliferation and migration of glioblastoma cells. These in vitro results support the rationale for conducting in vivo studies combining CA with 2-DG and SO in human glioblastomas.

KEYWORDS:
Glycolysis, Mango Ginger, ATP, Lactate, Gene Expression, Glioblastoma

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1. INTRODUCTION

Cancer cells, unlike normal cells, in general display a preference for aerobic glycolysis and reduced mitochondrial oxidative phosphorylation for glucose metabolism, a phenomenon known as the Warburg effect (Brown, 1962, Vander Heiden et al., 2009, Warburg, 1956). Cancer cells demonstrate high uptake of glucose and are more dependent on aerobic glycolysis to produce ATP for growth and maintenance. Since therapeutic selectivity or preferential killing of cancer cells without significant toxicity to normal cells is one of the most important considerations in cancer chemotherapy, targeting this metabolic pathway offers the potential for a selective approach to cancer treatment. Glioblastoma is the most common primary brain tumor in adults with an incidence rate of 3.19 per 100,000 persons in the United States. It is one of the most aggressive, lethal and incurable human tumors with a survival rate of 12-15 months in patients undergoing standard of care treatment involving surgery, chemotherapy and radiation therapy. Like the majority of other cancers, glioblastoma has been shown to undergo metabolism of glucose preferentially through aerobic glycolysis (*ie.*Warburg effect) unlike normal glial cells(Oudard et al., 1996a, Seyfried et al., 2014). These cells also metabolize ketone bodies poorly for energy, and withdrawal of glucose has induced

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apoptosis at rates dramatically higher than in normal human astrocytes(Maurer et al., 2011, Oudard et al., 1996b). Unlike oxidative phosphorylation, glycolysis is not an efficient mechanism for the production of ATP; however, it is an effective mechanism for *(i)* shunting carbons toward biosynthetic pathways necessary to drive cellular proliferation and *(ii)* generating the redox potential necessary to scavenge excess reactive oxygen species (ROS) ensuring cancer cell viability. Cellular reprogramming of glucose metabolism to fuel tumor cell growth has been shown to be driven by the Akt/Phosphoinositide 3-kinase (PI3 K)/mammalian target of rapamycin (mTOR) pathway, which is commonly activated in gliomas(Jelluma et al., 2006, Zhou et al., 2011).Several glycolytic inhibitors such as 2-deoxy-D-glucose (2-DG) and sodium oxamate (SO) have been investigated either alone or in combination with cancer drugs for their eventual use in cancer chemotherapy(Ben Sahra et al., 2010, Liu et al., 2001, Manerba et al., 2015, Maschek et al., 2004, Miskimins et al., 2014, Zhai et al., 2013). 2-DG blocks the first step in glycolysis by inhibiting hexokinase, the first rate-limiting enzyme involved in the conversion of glucose to glucose-6-phosphate(Brown, 1962, Lampidis et al., 2006, Pelicano et al., 2006). It is a sugar analog that interferes with glycolysis and glycosylation and has been shown to induce *in vitro* and *in vivo* antitumor effects in combination with chemotherapy(Boutrid et al., 2008, Datema and Schwarz, 1979, Kurtoglu et al., 2007a, Kurtoglu et al., 2007b, Maschek et al., 2004). SO is a glycolysis inhibitor targeting the lactate dehydrogenase –A (LDH-A) involved in the conversion of pyruvate to lactate in the cellular glycolysis pathway(Hua et al., 2014). By reducing pyruvate to lactate, LDH allows the rapid re-oxidation of NADH needed for sustaining glycolysis flux and assuring ATP synthesis and biomass production. Inhibition of LDH by oxamate causes a decrease in lactate production and suppression of cancer cell proliferation(Li et al., 2013, Yang et al., 2014). Due to its structural and chemical similarity to pyruvate, SO also inhibits pyruvate feeding into oxidative phosphorylation thereby also slowing down this energy pathway(Elwood, 1968). Thus, although both 2-DG and SO inhibit glycolysis they clearly do it via different mechanisms and also contain unique properties that affect other metabolic pathways differently. Recently, we have shown that the supercritical CO_2 extract of mango ginger (CA) has significant cytotoxic effects against human rhabdomyosarcoma and glioblastoma cells *in vitro* and *in vivo*. Furthermore, CA could be combined with conventional cancer drugs like vinblastine, cyclophosphamide, temozolomide, etoposide and irinotecan yielding synergistic cytotoxic effects (Ramachandran et al., 2015a, Ramachandran et al., 2015b, Ramachandran et al., 2017, Ramachandran et al., 2015c). Similarly glycolytic inhibitor 2-DG has been shown to operate through the AMPK/mTOR signaling pathway for its anticancer effects (Estan et al., 2012, Liu et al., 2016). In the present study, we investigated the effects of CA when combined with two glycolytic inhibitors (2-DG and SO) against the U-87MG human glioblastoma cell line.

2. MATERIALS AND METHODS

2.1. Cell line and Cell Culture

Human glioblastoma cell line (U-87MG) was purchased from American Type Culture Collection, Manassas, VA and the cells were grown in RPMI medium supplemented with 10% fetal bovine serum (FBS) and antibiotics in a humidified 5% CO_2 incubator.

2.2. Drugs

Both 2-DG (99% purity) and SO (98% purity) were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO. Supercritical CO_2 extract of mango ginger (*Curcuma amada -CA) was* prepared by Flavex Naturextrakte GmbH, Rehlingen, Germany. The usual yield of extract is 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 caryophyllene 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). Since CA is a supercritical CO_2 extract containing several compounds in it, dilution of CA was prepared in mg/ml concentrations. Similarly dilutions of 2-DG and SO in phosphate buffered saline (PBS) were prepared in mg/ml concentrations to match the CA dilutions.

2.3. Cytotoxicity

Glioblastoma cells were treated with increasing concentrations of CA alone or in combination with 2-DG and SO in low glucose RPMI medium 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., 2015a, Ramachandran et al., 2015c).

2.4. CompuSyn Analysis

To determine the synergistic/additive/antagonistic effects between drugs (CA, 2- DG and SO), cytotoxicity data was analyzed further using CompuSyn software (CompuSyn-Inc., Paramus, NJ). This program is based on Chou and Talalay's multiple drug effect equations and it defines synergism as a more-than expected additive effect and antagonism as a less-than expected additive effect(Chou and Talalay, 1983). The combination index was calculated by the Chou-Talalay equations for multiple drug effects, which take into account both potency (inhibitory concentration values) and shape (slope, m) of dose-effect curve (Chou and Talalay, 1983, Kapadia et al., 2013).

2.5. ATP inhibition

Increased glucose consumption and the dependency on glycolysis for ATP generation are hallmarks of cancer cells. Therefore, we have analyzed ATP levels in glioblastoma cells treated with CA, 2-DG and/or SO. U-87MG human glioblastoma cells (10^4/100 µl medium/well) were plated in 96-well plates and incubated overnight at 37°C in a CO_2 incubator. On the next day, cells were treated with increasing concentrations of CA, 2-DG and/or SO for 5 h in the CO_2 incubator. The plates were kept for 10 min at room temperature and 100 µl 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 µl of sample was transferred to a fresh 96-well plate for measuring the luminescence 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 are plotted.

2.6. Lactate Inhibition

U-87MG human glioblastoma cells (10^5/2 ml/well) were plated in 24-well plates and treated with increasing concentrations of CA, 2-D and/or SO followed by incubation at 37°C for 24 h in a CO_2 incubator. On the next day, 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 votexed well and kept at 4°C for 5 min. and centrifuged for 10 min at 1500 xg. About 25 µl of the extract was combined with the 1.475 l of solution containing 10 ml nicotinamide adenine dinucleotide hydrate (NADH), 2 ml glycine buffer, 4 ml sterile H_2O and 100 U lactate dehydrogenase at room temperature for 30 min. The plates were 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.

2.7. Gene Expression Studies by RT-PCR Assay

U-87MG glioblastoma cells ($5x10^6$ /5ml) were treated with CA, 2-DG and/or SO along with 150 µM CoCl2 at 37°C for 72 h. $CoCl_2$ treatment is included in the 2-DG and SO combinations to activate the genes associated with apoptosis and cell migration. The mRNA expression of genes associated with apoptosis (*Bax, Bcl-2, BNIP3, p21, p53, caspase3,*) and cell migration (*HIF-1α*, *MMP2, TIMP1*) were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) (Oudard et al., 1966b, Pelicano et al., 2006). The mRNA expression of a housekeeping gene, Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) 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.

2.8. Western blot analysis

U-87MG cells ($5 x10^6$/5 ml) were treated with increasing concentration of CA, 2-DG and/or SO for 72 h and total cellular protein was extracted using 0.5 ml of protein extraction buffer (Invitrogen Corporation, Frederick, CA). For 2-DG and SO combinations, cells were co-treated with $CoCl_2$ (150 µg/ml) for activation of genes associated with apoptosis and cell migration. The protein concentration was determined and 100 µg protein was separated on 7.5% SDS-PAGE. The separated protein was blotted on to a nitrocellulose filter. The filters were hybridized with anti-human monoclonal/polyclonal antibodies specific for each protein (Bax, Bcl-2, BNIP3, p21, p53, caspase-3, MMP-2, TIMP1 and 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 (Ramachandran et al., 2015b, Ramachandran et al., 2017)

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 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 Dunnett's comparison test (GraphPad Prism software, La Jolla, CA). The protein expression data was also statistically analyzed by 1-way analysis of variance with Dunnett's multiple comparison test (GraphPad Prism software, La Jolla, CA) (*p <0.05; **p <0.01; ***p <0.001).

3. RESULTS

3.1. Synergistic effects on cytotoxicity

The dose-effect curve presented in **Fig.1** A showed that SO was not cytotoxic up to a concentration of 5000 g/ml unlike CA and 2-DG and that CA is highly cytotoxic compared to 2-DG **(Table 1).** The 2-DG+SO combination failed to show any discernible increase in cytotoxicity compared to 2-DG alone. However, when CA is combined with 2-DG and/or SO, an enhanced cytotoxic effect is observed. The dose-effect plot and median effect plots for single agents as well as combinations indicate that this potentiation is highly significant. The combination index values for 2-DG+SO, CA+2-DG and CA+2-DG+SO combinations are below <1.0 at IC_{50}, IC_{75} and IC_{90} levels demonstrating the synergism between

these agents **(Table 2).**
The drug reduction index (DRI) values show that the concentration of glycolytic inhibitors can be reduced substantially when combined with CA in order to achieve comparable IC_{50}, IC_{75} or IC_{90} levels of cell death, and the same applies when considering the amount of CA required to induce cell death with and without addition of 2-DG or SO.

3.2. ATP inhibition

An elevated rate of glucose consumption and the dependency on aerobic glycolysis for ATP generation are noticeable characteristics of cancer cells. Therefore, ATP levels were analyzed in U-87MG glioblastoma cells treated with CA, 2-DG and/or SO. Low concentrations of 2-DG (up to100 g/ml) inhibit ATP synthesis up to 40% which also can be achieved similarly by low levels of SO **(Fig. 2).** However, when these two agents are combined, there is a 65% reduction in ATP at 50 µg/ml and 100 µg/ml concentrations.

Titrating the levels of ATP inhibition by CA shows that at 10 g/ml, 70% reduction is achieved and that ATP is inhibited by 90% at 50 g/ml while at a100 g/ml of CA, ATP levels become undetectable **(Fig.3).** These marked decreases in ATP are also achieved when CA is combined with 2-DG at lower CA concentrations (at doses that 2-DG alone has very low effect on ATP) i.e., 95 % reduction of ATP at 20 µg/ml CA and complete inhibition at 45 µg/ml CA+2-DG.

3.3. Lactate inhibition

Since lactate dehydrogenase is involved in the conversion of pyruvate to lactate in the glycolytic process, elevated lactate concentration is usually noticed in cancer cells. Therefore, lactate concentrations were analyzed in U-87MG glioblastoma cells treated with CA, 2-DG and/or SO. Both 2-DG and SO inhibit lactate synthesis significantly at concentrations >100 µg/ml in glioblastoma cells **(Fig. 4).** 2-DG is a more efficient inhibitor than SO with 75% inhibition at 200 g/ml. Also, 2-DG + SO combination has almost similar levels of inhibition as 2-DG. Furthermore, we noticed a plateauing effect by 2-DG and 2-DG+SO at concentrations >200 g/ml. Being a lactate dehydrogenase A inhibitor, the highest concentration of 1000 g/ml of SO appeared to be better than the similar concentration of 2-DG on lactate inhibition. Since CA is highly cytotoxic at low concentrations, we have used much lower concentrations of CA and 2-DG for lactate inhibition studies **(Fig. 5).** CA is a more potent inhibitor of lactate than 2-DG reducing lactate by approximately 70% at 100 µg/ml. On the other hand, 2-DG was able to inhibit only about 43% lactate at a similar concentration. When CA was combined with 2-DG, increased lactate inhibition is observed at 100 µg/ml concentration as compared to that of single agents.

3.4. mRNA expression

To understand the mechanism of action of mango ginger extract and glycolytic inhibitors alone as well as in combination, gene expression studies were performed using RT-PCR analysis and western blot hybridization. RT-PCR analysis of apoptosis and metastasis associated genes in 2-DG, SO and 2-DG+ SO treated glioblastoma cells are given in **Fig. 6a** and the quantification of expression levels (fold) is presented in **Fig. 6b.** Unlike Bax mRNA expression which was unchanged at varying concentrations of 2-DG and SO, Bcl-2 mRNA level was significantly down regulated compared to untreated and $CoCl_2$ treated controls by these glycolytic inhibitors individually and when combined resulted in the increase of Bax/Bcl-2 ratio (data not shown). P21 mRNA expression was up regulated by 2-DG and the combination treatment. While mutant p53 was down regulated by higher doses of 2-DG and SO individually, TIMP1 mRNA expression was up regulated by SO and 2-DG combination.

The changes in mRNA expression of genes induced by CA, CA+2-DG and CA+2-DG +SO combination are given in **Fig. 7a** and the quantification of expression (fold level) is given in **Fig. 7b.** Again even when either 2-DG or SO is combined with CA Bax mRNA expression is unaffected by drug treatment. However, Bcl-2 mRNA expression is down regulated significantly by CA, 2-DGand/or SO, and the triple combination showing more inhibition. Similarly, BNIP3 and mutant p53 mRNA was down regulated by CA, CA+2-DG and CA+2-DG+SO combination.

3.5. Protein expression

Western blot hybridization was performed to analyze the translational products of apoptosis and metastasis genes, and to elucidate the mechanism of action of glycolytic inhibitors and CA individually as wells as in combination in glioblastoma cells. The results of protein expression analyzed in glioblastoma cells treated with 2-DG and/or SO are presented in **Fig. 8a** and their respective quantification is shown in **Fig. 8b.** Surprisingly, unlike at mRNA level, Bax protein levels increase significantly with treatment of cells with 2-DG, SO and 2G+SO. On the other hand as expected, Bcl-2 protein levels decreased significantly with 2-DG (higher concentrations), SO and 2-DG+SO treatments. Higher concentrations of 2-DG, SO and 2-DG+SO also down regulate BNIP3 protein. While higher doses of SO and 2-DG+SO down regulate mutant p53 protein expression, they up regulate p21 protein expression. Similarly, caspase-3 protein expression is up regulated by 2-DG, SO and 2-DG+SO treatment in glioblastoma cells.

The changes in protein expression in glioblastoma cells treated with CA, CA+2-DG and CA+2-DG+SO are presented in **Fig. 9a** and their respective quantification in **Fig. 9b.** CA+2-DG at higher doses and CA+2-DG +SO at all selected doses significantly down regulated Bcl-2 protein expression. On the other hand, CA, CA+2-DG and CA+2-DG +SO at higher doses have up regulated Bax protein expression. CA at 50 g/ml, CA+2-DG and CA+2-DG+SO at higher doses have down regulated mutant p53 protein expression. CA+2-DG and CA+2-DG+SO have up regulated p21 expression significantly. Treatment of

glioblastoma cells with CA, CA+2-DG and CA+2-DG +SO have up regulated the caspase-3 expression, with combinations causing better increase. Of the two biomarkers associated with metastasis, CA at high doses of >20 g/ml, CA+2-DGand CA+2-DG+SO down regulates MMP2 and up regulates TIMP1 protein expression.

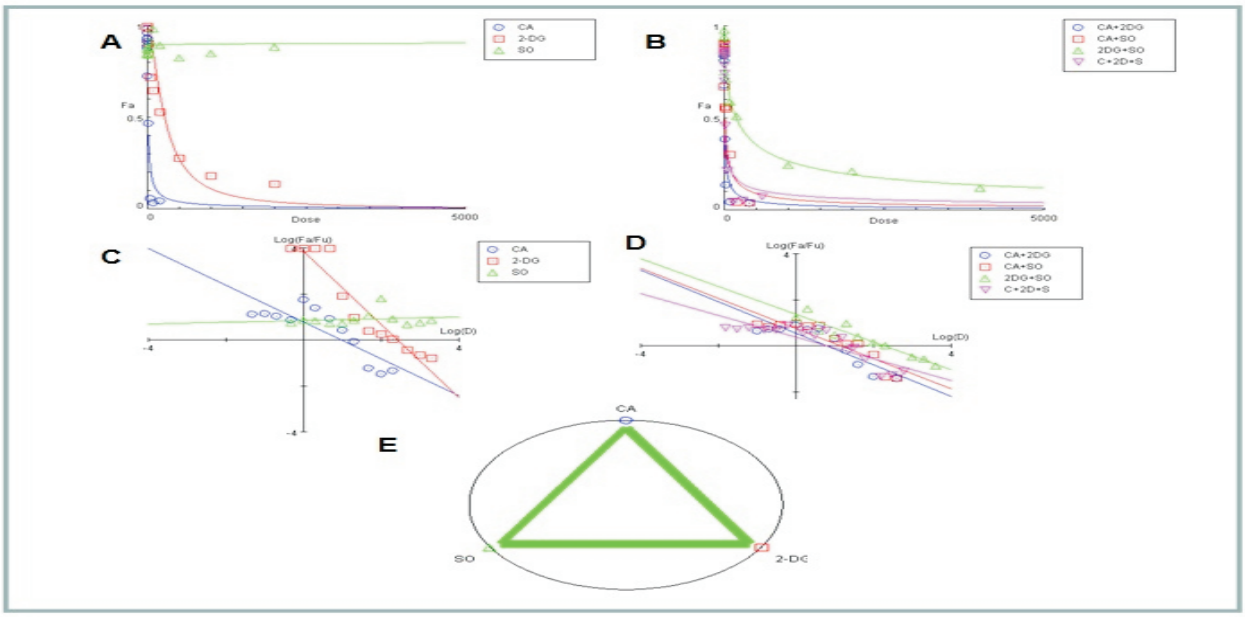

Figure 1. CompuSyn analysis of cytotoxicity data to determine synergism/additive effect/antagonism between supercritical CO_2 extract of mango ginger (CA) and glycolytic inhibitors (2-DG and SO) in U-87MG glioiblastoma cell line. (**A&C**) Dose-effect and median effect plots of single agents (supercritical extract of mango ginger [CA], 2-deoxy-D-glucose (2-DG) and sodium oxamate (SO). (**B&D**) Dose-effect and median-effect plots of extract and glycolysis inhibitor combinations [CA+2-DG, CA+SO, 2-DG+SO and CA+2-DG+SO(C+2D+S)].

Table 1. Cytoxicity of supercritical extract of mango ginger (CA) and glycolysis inbibitors (2-DG and SO) in U-87MG glioblastoma cell line

Drug	IC_{50} (µg/ml)	IC_{75} (µg/ml)	IC_{90} (µg/ml)
CA	8.20	14.31	21.30
2-DG	198.50	645.83	>5000
SO	>5000	>5000	>5000
CA+2-DG	2.61	8.20	15.11
CA+SO	7.62	13.15	19.40
2-DG+SO	207.00	562.00	>5000
CA+2-DG+SO	7.60	12.77	15.11

CA, supercritical extract of mango ginger; 2-DG, 2-deoxy-D-glucose; SO, sodium oxamate

Table 2. Combination Index (CI) and Drug Reduction Index estimates (DRI) between supercritical extract of mango ginger (CA) and glycolysis inhibitors (2-deoxy-D-glucose and sodium oxamate) in U-87MG glioblastoma cell line

Combination	CI value at IC_{50}	CI value at IC_{75}	CI value at IC_{90}	DRI –CA IC_{50}	DRI-2-DG IC_{50}	DRI-SO IC_{50}
CA + 2-DG	0.76	0.70	0.64	1.31	1728.27	-
CA+SO	0.57	0. 42	0.32	1.76	-	1370.74
2-DG+SO	3.39	1.24	0.95	-	0.80	0.47
CA+2-DG+SO	0.56	0.59	0.64	1.79	2318.90	1395.38

CA, supercritical extract of mango ginger; 2-DG, 2-deoxy-D-glucose; SO, sodium oxamate
CI, a quantitative measure of the degree of drug interaction in terms of synergism and antagonism for a given endpoint of the effect measurement (Chou and Talalay 1983); DRI, a measure of how many folds the dose of CA, 2-DG or SO may be reduced at a given effect level when compared with the doses of each alone.

CI values

0.1-0.3 = strong synergism
0.3-0.7 = synergism
0.8-0.9 = moderate to slight synergism
0.9-1.1 = nearly additive
1.1-1.45 = moderate to slight antagonism

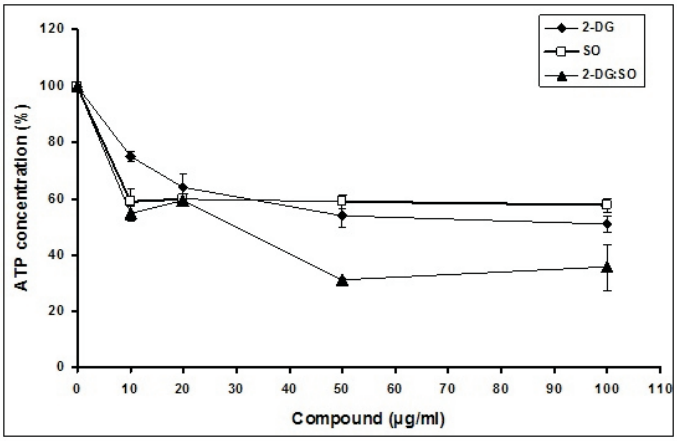

Figure 2. Effect of 2-deoxy-D-glucose (2-DG), sodium oxamate (SO) and their combination on inhibition of ATP synthesis in U-87MG glioblastoma cell line.

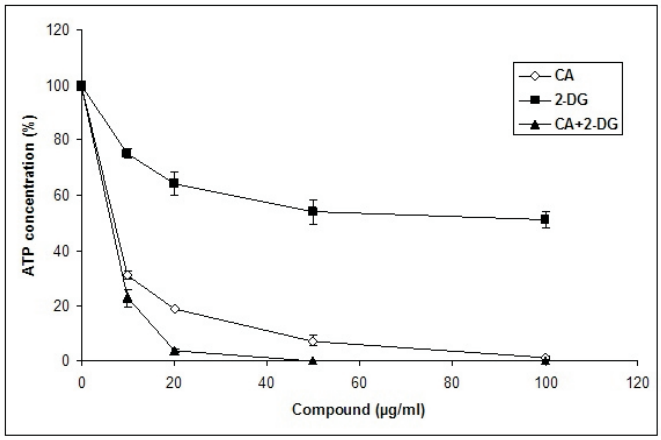

Figure 3. Effect of supercritical CO_2 extract of mango ginger (CA), 2-deoxy-D-glucose (2-DG) and their combination on inhibition of ATP synthesis in U-87MG glioblastoma cell line.

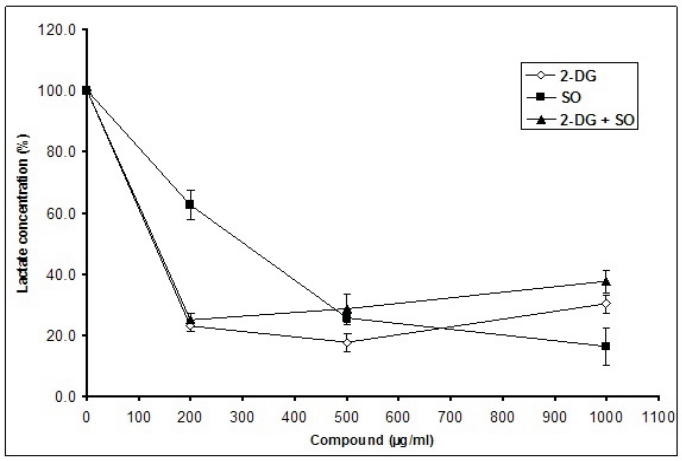

Figure 4. Effect of glycolytic inhibitors [2-deoxy-D-glucose (2-DG), sodium oxamate (SO)] on inhibition of lactate synthesis in U-87MG glioblastoma cell line.

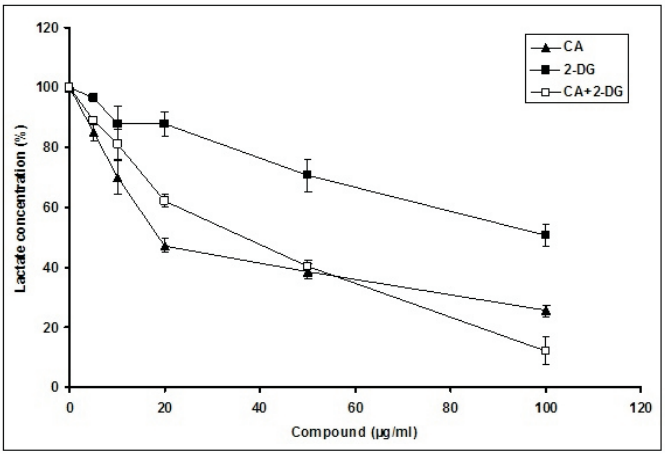

Figure 5. Effect of supercritical CO_2 extract of mango ginger (CA), 2-deoxy-D-glucose (2-DG) and their combination on inhibition of lactate synthesis in U-87MG glioblastoma cell line.

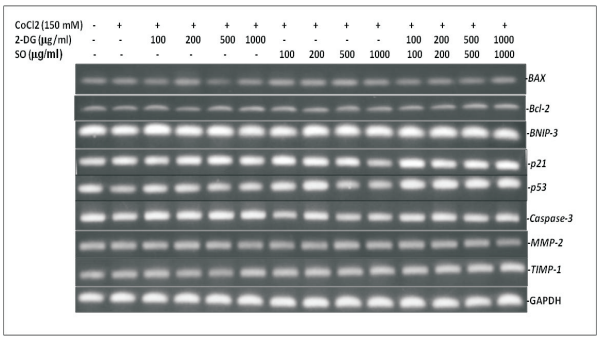

Figure 6a. Effect of 2-DG and/or SO on mRNA expression of genes associated with apoptosis and metastasis in U-87 MG glioblastoma cell line analyzed by RT-PCR assay.

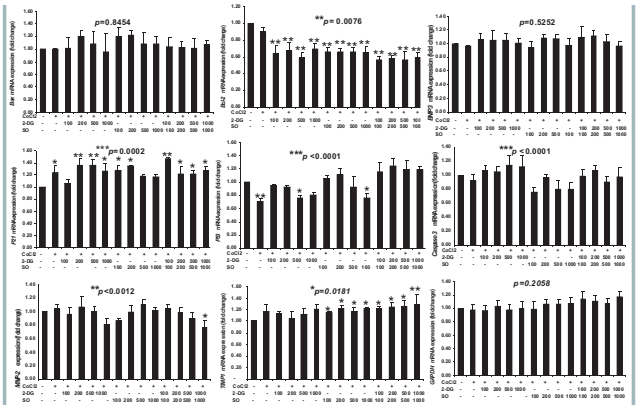

Figure 6b. Quantification of mRNA expression of genes associated with apoptosis and metastasis in U-87 glioblastoma cell line treated with 2-DG and/or SO (g/ml) with or without $CoCl_2$ (150 M). The relative expression of genes (average pixel units) is plotted against drug concentrations. The significant difference between treatments was compared by 1-way analysis of variance with Dunnett's multiple comparison test (GraphPad Prism software, La Jolla, CA) (*p <0.05; **p <0.01; ***p <0.001).

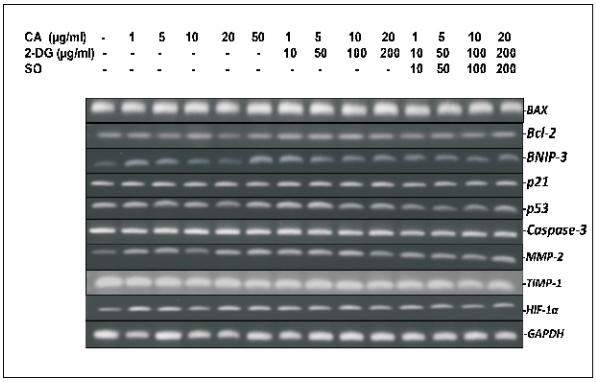

Figure 7a. Effect of supercritical CO_2 extract of mango ginger (CA), 2-deoxy-D-glucose (2-DG) and their combination on mRNA expression of genes associated with apoptosis and metastasis in U-87 MG glioblastoma cell line analyzed by RT-

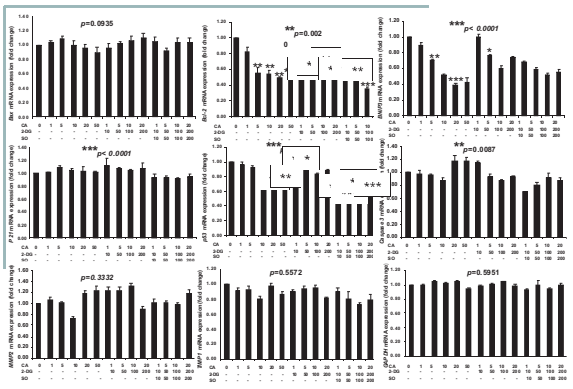

Figure 7b. Quantification of mRNA expression of genes associated with apoptosis and metastasis in U-87 glioblastoma cell line treated with CA and/or 2-DG. The relative expression of genes (average pixel units) is plotted against drug concentrations. The significant difference between treatments was compared by 1-way analysis of variance with Dunnett's multiple comparison test (GraphPad Prism software, La Jolla, CA) (*p <0.05; **p <0.01; ***p <0.001).

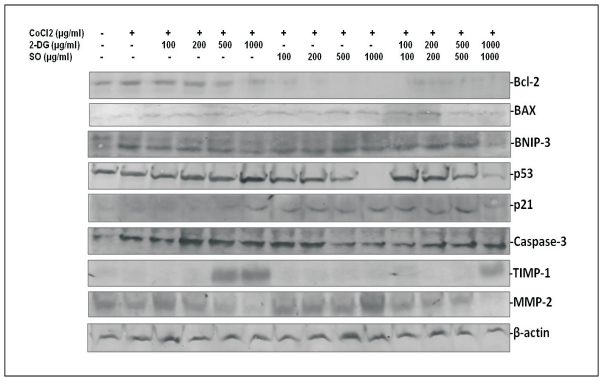

Figure 8a. Effect of 2-DG and/or SO on expression of proteins biomarkers associated with apoptosis and metastasis in glioblastoma cell line analyzed by western blot hybridization.

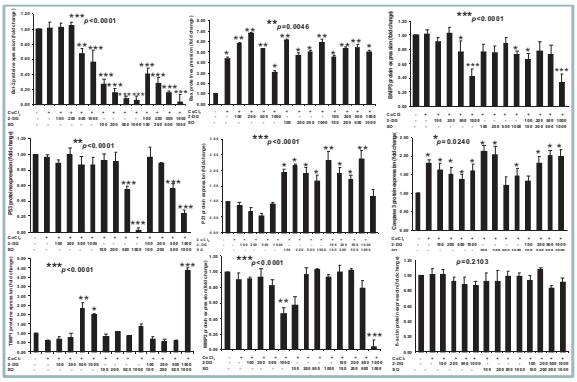

Figure 8b. Quantification of expression of protein biomarkers (western blots) associated with apoptosis and metastasis in glioblastoma cell line treated with 2-DG and/or SO. The relative expression of genes (average pixel units) is plotted against drug concentrations. The significant difference between treatments was compared by 1-way analysis of variance with Dunnett's multiple comparison test (GraphPad Prism software, La Jolla, CA) (*p <0.05; **p <0.01; ***p <0.001).

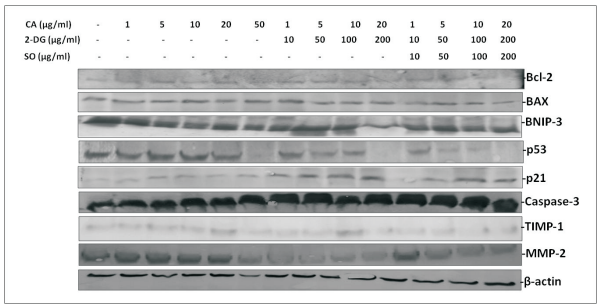

Figure 9a. Effect of CA, 2-DG and/or SO on expression of protein biomarkers (western blot) associated with apoptosis and metastasis in U-87MG glioblastoma cell line analyzed by western blot hybridization.

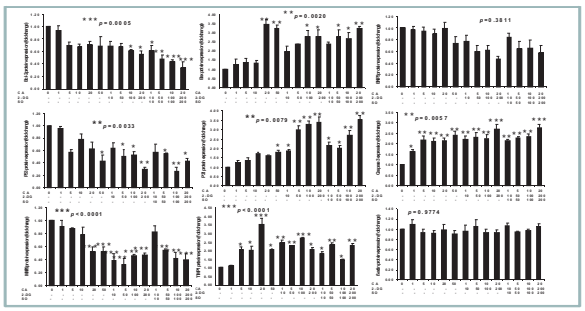

Figure 9b. Quantification of expression of protein biomarkers (western blots) associated with apoptosis and metastasis in glioblastoma cell line treated with CA, 2-DG and/or SO. The relative expression of genes (average pixel units) is plotted against drug concentrations. The significant difference between treatments was compared by 1-way analysis of variance with Dunnett's multiple comparison test (GraphPad Prism software, La Jolla, CA) (*p <0.05; **p <0.01; ***p

4. DISCUSSION

Despite recent progress, glioblastoma remains an incurable disease with survival under 15 months for most patients. Selective killing of cancer cells without significant toxicity to normal cells is one of the most important considerations for cancer chemotherapy. Most cancer cells exhibit increased glycolysis and use this metabolic pathway for generation of ATP as a main source of their energy supply. This phenomenon is known as Warburg effect and is considered as one of the most fundamental metabolic alterations during malignant transformation. Because of this specific alteration, compounds that inhibit glycolysis and other relevant metabolic processes have been investigated for their use in cancer treatment(Ben Sahra et al., 2010, Maschek et al., 2004, Pelicano et al., 2006, Wang et al., 2012). 2-DG is a glucose analog and has been known to act as a competitive inhibitor of glucose metabolism (Brown, 1962). However the effectiveness of 2-DG is significantly affected by the presence of glucose and as we have found here only partially reduces ATP and lactate production indicating that either the concentrations used were not sufficient to completely block glycolysis or that other fuels and pathways such as glutaminolysis and or fatty acid oxidation could act as alternative energy sources(Liu et al., 2001, Liu et al., 2002, Maher et al., 2004, Xi et al., 2014). 2-DG has also been shown to enhance the anticancer activity of adriamycin and paclitaxel in mice bearing human osteosarcoma and non-small cell lung cancer xenografts(Maschek et al., 2004). A clinical trial suggests that 2-DG at doses up to 250 mg/kg appears safe for use in combination with radiation therapy in patients with glioblastoma multiforme(Xi et al., 2014).

In the glycolysis pathway, lactate dehydrogenase (LDH), a key regulator, reversibly catalyzes the conversion of pyruvate to lactate. Recently, oxamate, an inhibitor of LDH, has been shown to be a promising anticancer agent (Liu et al., 2015, Zhao et al., 2015). In doxorubicin-resistant chondrosarcoma cells, oxamate enhances sensitivity to doxorubicin (Hua et al., 2014). The inhibition of LDH-A by oxamate induces G2/M arrest, apoptosis and increased radiosensitivity in carcinoma cells (Yang et al., 2014, Zhai et al., 2013). It is also reported that LDH-A plays an important role in taxol resistance and inhibition of LDH-A by oxamate re-sensitizes taxol-resistant cells to taxol(Zhou et al., 2010). Recently we showed that CA is highly cytotoxic to U-87MG glioblastoma cells and described the underlying mechanisms associated with the increased cytotoxicity of this natural product. The major active ingredient (E)-labda-8(17), 12-diene-15,16-dial (LDD) which is about 61.7% of CA appeared to be responsible for the increased cytotoxicity of CA in glioblastoma cells (Ramachandran et al., 2015a, Ramachandran et al., 2015b, Ramachandran et al., 2015c). Since CA with glycolytic inhibitors have similar targets, in this investigation we examined the combined anticancer effect of CA with glycolytic inhibitors like 2-DG and SO.

Among the two glycolytic inhibitors SO failed to show any cytotoxic effect in glioblastoma cells. The IC values for 2-DG were also high, indicating the need to combine it with other anticancer agents. CA is highly cytotoxic to glioblastoma cells based on the IC values. However, when combined with 2-DG and/or SO, decrease in the IC value of CA was observed indicating the potentiation of the agents. CompuSyn analysis of cytotoxic values showed that the CA+2-DG, CA+SO and CA+2-DG +SO combinations are synergistic for cell killing. Interestingly, the 2-DG +SO combination on the other hand is antagonistic. Sahra et al. (2010) reported that treatment of prostate cancer cells with 2-DG and metformin induces p53- and AMPK-dependent apoptosis and exerts an additive antiproliferative effect compared with either drug(Ben Sahra et al., 2010). Furthermore, metformin inhibits 2-DG-induced autophagy, decreases bectin-1 expression and triggers a switch from the survival process to cell death. It is also reported that combination of herceptin (trastuzumab) and 2-DG, or oxamate, synergistically inhibited the growth of both trastuzumab-sensitive and -resistant breast cancer cells *in vitro* (Zhao et al., 2015). A similar synergistic effect has been observed with a combination of taxol and oxamate in taxol-resistant cancer cells promoting apoptosis in these cells (Zhou et al., 2010). Among the three agents, CA has the highest inhibitory effect on ATP synthesis followed by 2-DG and SO. The alterations in ATP concentration with increase in treatment doses do not correspond exactly with the cytotoxicity and it is quite possible that other cell death mechanisms that do not involve alterations in ATP concentration may be playing a role. However, the combination of CA+2-DG has a significantly higher effect on ATP inhibition than either agent alone, which correlates well with the cytotoxicity data. Similar to ATP inhibition, CA induced the highest inhibition of lactate dehydrogenase at low concentrations of <50 μg/ml compared to 2-DG and SO treatment. Similar to ATP inhibition, we have observed an increase in lactate inhibition when CA and 2-DG are combined at high dose of 100 μg/ml. Therefore, enhancement of glycolysis inhibition is possible by combining CA with glycolytic inhibitors in glioblastoma.

Analysis of mRNA expression showed that the glycolytic inhibitors down regulate the expression of anti-apoptotic markers Bcl-2 and mutant p53 and up regulate pro-apoptotic marker p21 as well as the anti-metastatic marker TIMP1. These markers were also correspondingly modulated at protein levels by 2-DG and SO. Additionally, Bax and caspase-3 protein expression were up regulated by both glycolytic inhibitors. In the CA combinations, while Bcl-2 and p53 protein expression was down regulated, Bax and p21 expression was up regulated correlating with death induced synergism when either of the glycolysis inhibitors were combined with CA. The CA+2-DG and CA+2-DG+SO combinations also induced up regulation of TIMP1 and down regulation of MMP2, which previously had been shown to regulate metastasis. Thus, our results suggest a possible inhibitory activity of metastasis when CA is combined with ether 2-DG or SO. The modulation in the expression of pro-apoptotic (Bax, caspase-3 and p21) and anti-apoptotic (Bcl-2, mutant p53) genes by CA, 2-DG and/or SO in U-87MG glioblastoma cells is noteworthy since it has a

direct relationship on apoptosis (Speirs et al., 2011). Bax is reported to counter the death repressor activity of Bcl-2 by Bax/Bcl-2 heterodimerization (Oltvai et al., 1993). In our earlier investigations with CA, we have indeed found that CA alone inhibits MMP2 and MMP9 activity correlating with inhibition of migration of tumor cells (Ramachandran et al., 2015c). It remains to be investigated whether the down regulation of MMP2 and up regulation of TIMP1 (tissue inhibitor of matrix metalloproteinase 1) by combination of CA and glycolytic inhibitors (2-DG and SO) observed in the current investigation, may lead to inhibition of cell invasion and metastasis in glioblastoma (Lu et al., 2014). In conclusion, the synergistic effects we find by combining CA with the glycolytic inhibitors 2-DG and/or SO *in vitro* may be beneficial for inhibiting growth, proliferation as well as migration of glioblastoma cells *in vivo* which supports the feasibility of further testing our findings in animal models of this disease.

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, Ms. Ashley Juan and Ms. Adriana M. Prado are also employees of Dharma Biomedical LLC.

ETHICAL APPROVAL

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

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ABBREVIATION(S)

2-DG: 2-Deoxy-D-glucose
Akt: Protein kinase B (PKB)
AMPK: 5'Adenosine monophosphate activated protein kinase
ATP: Adenosine triphosphate
Bax: Bcl-2-associated X protein
Bcl-2: B-cell lymphoma-2
BNIP3, Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3
CA: Supercritical CO2 extract of mango ginger
CoCl2: Cobalt chloride
FBS: Fetal bovine serum
GC-MS: Gas chromatography-mass spectrometry
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
HIF-1α: Hypoxia inducible factor 1α
HPLC: High-performance liquid chromatography
IC: Inhibitory concentration
LDD: (E)-labda-8(17),12 dien-15,16 dial
LDH A: Lactate dehydrogenase-A
MMP2: Matrix metalloproteinase 2
mTOR: Mammalian target of rapamycin
MTT: [2-(4,5-Dimethyl thiazol-2-yl)-2,5-Diphenyltetrazolium bromide]
NADPH: Nicotinamide adenine dinucleotide hydrate
p21: Cyclin-dependent kinase inhibitor 1
p53: Tumor protein 53
PI3K: Phosphoinositide 3-kinase
ROS: Reactive oxygen species
RPMI: Roswell Park Memorial Institute
RT-PCR: Reverse transcription-polymerase chain reaction
SO: Sodium oxamate
TIMP1: Tissue inhibitor of metalloproteinase

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