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RESEARCH ARTICLE

Anti cancer and Anti -metastatic E ffects of Supercriti cal E xtracts of Hops (Humulus lupulus L.) and M ango ginger (Curcuma amada Roxb.) in H uman G lioblastoma

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Abstract

Glioblastoma is one of the most aggressive, lethal and in curable primary brain tumors with a dismal prognosis in humans. Mango ginger (*Curcuma amad*a) and hops *(Humulus lupulus*) are two botanicals containing phytochemicals with potential anticancer effects. We have investigated the anticancer and antimetastatic properties of supercritical CO_2 extract of mango ginger (CA) and ethanol extract of hops (HL) in the U-87MG human glioblastoma cell line. Both CA and HL individually demonstrate strong cytotoxicity against glioblastom a cells. CompuSyn analysis of cytotoxicity data confirms that CA and HL are synergistic for cytotoxicity with combination index (CI) values of <1.0. Additionally, CA and HL individually as well as the combination significantly inhibit MMP-2 and MMP-9 activity, tumor cell migration (transendothelial cell migration assay) and AKT phosphorylation in U-87 MG cells. CA and HL inhibit glycolysis in U-87MG cells as indicated by the inhibition of ATP and lactate synthesis with the CA+HL combination demonstrating strong inhibition of glycolysis via the reduction of ATP and lactate synthesis compared to cells treated by each extract alone. CA and HL treatment down regulates the expression of proteins associated with metastasis, MMP-2 and MMP-9 and up regulates the expression of TIMP1. Proteins associated with apoptosis, inflammation and energy metabolism were also modulated by CA and HL treatment of glioblastoma cells. These results suggest that CA and HL can be combined for the therapeutic management of glioblastomas.

Keywords: glycolysis; Warburg effect; hops; mango ginger; cell migration

Introduction

Glioblastoma is the most common primary malignant brain tumor in adults with an incidence rate of 3.19 per 100,000 persons in United States. It is one of the most aggressive, lethal and incurable human tumors with a survival time of 12-15 months in patients undergoing standard of care treatment involving surgery, chemotherapy and radiation therapy. Generally, chemotherapy options for high-grade gliomas are very much limited due to the poor penetration of the blood-brain barrier and/or drug resistance of tumor cells [1].

Hops (*Humulus lupulu*s L) is a dioecious, perennial plant belonging to the Cannabaceae family, which is a raw material of beer that serves as an important source of phenolic compounds imparting stability, bitter taste and flavor to the beer. The use of hops as a medicinal plant has more than 2000 years of history [2] . The ancient healers used hops against leprosy, foot odor, constipation, and for blood purification [3] . Hops have been consid-

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ered to be an important source of substances having estrogenic effects and therefore, they are common components of commercial dietary supplements for women with menopausal symptoms or hormonal imbalances [4, 5] . The polyphenols, essential oils, bitter acids (a and β-bitter acids) and the various compounds in each of these constituents in hops are reported to possess antioxidant, anti-inflammatory, antimicrobial, anticancer and antiangiogenic properties [3, 5–9] .

The dried hops contain 14% polyphenols, mainly phenolic acids, prenylated chalcones, flavonoids, catechins and proanthocyanidins [10–12]. In addition, hops provide a resin consisting of bitter compounds such as humulones and lupulones [10, 13] . Prenylflavonoids, a subclass of flavonoids with restricted distribution in the plant kingdom but present in hops, display diverse molecular activities including distinct anti-proliferative activity towards human cancer cells and suppression of cancer cell invasion [8, 14] . Two interesting prenylflavonoids with antiproliferative activity are 6-prenylnaringenin (6-PN) and 8-prenylnaringenin (8-PN). Their common precursor compound xanthohumol (XN), a prenylated chalcon, is the principal prenylflavonoid in the female flowers of hop plant and provides 80-90% of its total flavonoid content [15] . During the beer brewery process XN can isomerize into isoxanthohumol (IX), which can subsequently be converted to 8-PN by gut bacteria. Additionally, XN precursor desmethoxyxanthohumol spontaneously isomerizes into 6-PN and 8-PN after addition of the hop cones into the boiling wort in the brew kettle [16] .

In our earlier investigations, we have reported extensively on the anticancer properties of CA, a proprietary supercritical CO_2 extract of mango ginger (*Curcuma amad*a Roxb.) both *in vitro* and *in vivo* in human glioblastoma cell lines [17-20]. CA can be combined with conventional cancer drugs like vinbalstine, cyclophosphamide, temozolomide, etoposide and irinotecan with synergistic cytotoxic effects [17–19] . The main objective of current study was to investigate anticancer properties of an ethanolic extract of hop s (HL) and synergetic effects with CA in human glioblastoma cells including evaluation of molecular signaling pathways related to inhibition of proliferation and invasion of glioblastoma cells.

Materials and methods

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% FBS and antibiotics in a humidified 5% CO_2 incubator.

Extracts

Supercritical CO_2 extract of mango ginger (CA) and an extract of hops (HL) prepared with 90% ethanol were obtained from 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 [20]. HL is a dark green, almost black pasty extract with typical hop smell. It is standardized to 33% humulones by addition of about 20% organic olive oil. HPLC analysis showed that it contains 32.3% humulones (alpha acids), 15.9% lupulones (beta acids) and 1.5% xanthohumol.

Cytotoxicity assay

Glioblastoma cells were treated with increasing concentrations of CA, HL or their combination in 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 [17, 18] .

CompuSyn Analysis

To determine the synergistic/additive/antagonistic effects between CA and HL, 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 defines synergism as a more-than expected additive effect and antagonism as a less-than expected additive effect [21] . The combination index was calculated by the Chou-Talalay 's equations for multiple drug effects, which take into account potency (inhibitory concentration values) and shape (slope, m) of dose-effect curve [21, 22].

Analysis of MMP activity by zymography

The gelatinases MMP-2 and MMP-9 are two members of the MMP family that have been studied extensively owing to their consistent association with tumor invasion and metastasis. To assess the MMP activity, tumor cells were treated with increasing concentrations of CA and/or HL for 72h and total protein was extracted using Invitrogen protein extraction buffer and 100

m g protein was separated on 10% SDS-PAGE containing 1% gelatin [17, 18] . The gel was incubated in renaturing solution for 30 min, followed by two incubations of developing solution 30 min each time, and then stained with coomassie blue stain for 1 h. The gels were destained overnight with destaining solution until areas of gelatinase activity appeared as clear sharp bands over the blue background [23]. The bands were quantified using Unscan software and plotted against CA and/or HL concentrations.

Tumor cell transendothelial migration assay

The calorimetric QCM tumor cell transendothelial cell migration assay kit (Millipore) was used to analyze the ability of tumor cells to invade the endothelium. To perform this assay, human vascular endothelial cells (HUVEC) cells (10^5 /250 ml/well of F15 medium supplemented with FBS and antibiotics) were plated in each Boydon chambers and allowed to grow for 72 h in the CO_2 incubator until the endothelial cells formed a monolayer. The endothelial cells were activated with 20 ng/ml recombinant human TNF-α for 18 h by incubating in a CO_2 incubator at 37°C. On the fifth day, U-87MG tumor cells were harvested and resuspended in serum-free RPMI medium containing only 0.5% FBS. The medium was removed from HUVEC cells growing in Boydon chambers and tumor cells (10^5 /well in 0.25 ml of serum free medium) were added into the Boydon chambers. The insert chambers were then transferred to wells containing 300 ml of serum free RPMI medium. Once the cells were settled (after 1 h) both insert chambers and wells containing serum free medium were treated with increasing concentrations of CA and/or HL (0-20 µg/ml). The multiwell plate containing HUVEC+U-87MG cells was incubated in a CO_2 incubator at 37°C for 24 h. The invasion of tumor cells across the endothelium is determined by measuring the number of cells that migrate to the lower chamber. The inner plate is stained with cell staining solution for 15 min. The excess stain was washed with distilled water and stain was extracted from migrated tumor cells using 200 µl of stain extraction solution. The absorbance of the extracted stain was measured at 450 nm in a plate reader and the inhibition (%) of tumor cell migration by CA and/or HL was estimated as compared to the untreated control sample [19] .

Analysis of AKT Phosphorylation

U-87MG glioblastoma cells (2x10^6 /5ml) was treated with increasing concentrations of CA and/or HL (0- 50 µg/ml) for 72 h and total cellular proteins were isolated using lysis buffer from the Cell Signaling Technology, Inc., Danvers, MA. The concentrations of protein in the lysate were estimated by the Lowrie's method. The phosphorylated form of AKT in the cellular lysate equivalent to 10 µg protein was analyzed using the Surveyor IC human phospho-AKT Pan Specific ELISA kit according to manufacturer's instructions (Cell Signaling Technology, Inc., Danvers, MA). The relative phospho-AKT levels of U-87MG cells under increasing concentrations of CA were plotted [19] .

ATP Assay

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 and/or HL 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 thoroughly 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 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 [24].

Lactate Assay

U-87MG human glioblastoma cells (2 x10^5 /ml/well) were plated in 24-well plates and treated with increasing concentrations of CA and/o r HL followed by incubation at 37°C in a CO_2 incubator. About 0.5ml 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 mixed well on a vortex 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 m l of working solution containing nictotinamide adenine dincleotide hydrate (NAD), glycine buffer and lactate dehydrogenase for 30 min at room temperature. 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 [24].

Western Blot Analysis

U-87MG cells (5 x10^6 /5 ml) were treated with increasing concentration of CA (0-100 µg/ml) and/or HL for 72 h and total cellular protein was extracted using 0.5 ml of Invitrogen protein extraction buffer (Invitrogen Corporation, Frederick, CA). The protein concentration was determined and 100 µg protein was separated on 7.5% SDS-PAGE. The separated protein was blotted on 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, MMP2, TIMP1

and β-tubulin 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^{TM} 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 [17–19].

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 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 Dunnett's comparison test (GraphPad Prism software, La Jolla, CA). The protein expression data was statistically compared with the expression of house keeping control gene (β-actin) using unpaired *t* test with Welch's correction and the significance level presented in the graph (*p <0.05, **p <0.01 and *** p <0.001).

Results

Cytotoxicity of CA, HL and CA+HL

The results of cytotoxic studies are presented in Fig. 1 and Table 1. Both CA and HL are strongly cytotoxic to glioblastoma cells with HL more cytotoxic than CA at the IC_{50} value (3.72 vs. 5.03 mg/ml respectively) and CA more cytotoxic at IC_{75} and IC_{90} values (12.87 and 37.14 vs. 22.93 and 43.87 mg/ml respectively). When the two extracts were combined there was significant reduction in IC values indicating synergism. The cytotoxic values for CA+HL combination are 2.70, 7.35 and 11.64 mg/ml at IC_{50}, IC_{75} and IC_{90} levels, respectively.

The compuSyn analysis of cytotoxicity was performed to determine the synergism/additive effect/antagonism between CA and HL. The dose-effect curve and median-effect plots presented in Fig. 2A and B show the potentiating effect of combination. The combination plot (Fig. 2C) and polygonogram (Fig. 2D) show the synergism between CA and HL in glioblastoma. Synergism is also illustrated by the combination index values given in Table 2. The CI values are all below 1 indicating the synergistic cytotoxic effect between CA and HL.

Matrix metalloproteinase activity

Zymograms indicating the effect of CA, HL and CA +HL are shown Fig. 3A and quantified activity data are presented in Fig. 3B. CA has a dose-dependent inhibition of MMP2 and MMP9 activity in glioblastoma cells. HL has a pronounced effect on MMP activities and shut down the MMP2 and MMP9 activity right from the lowest concentration of 2 m g/ml onwards. The combination has similar inhibitory effect on MMP2 and MMP9 as HL alone.

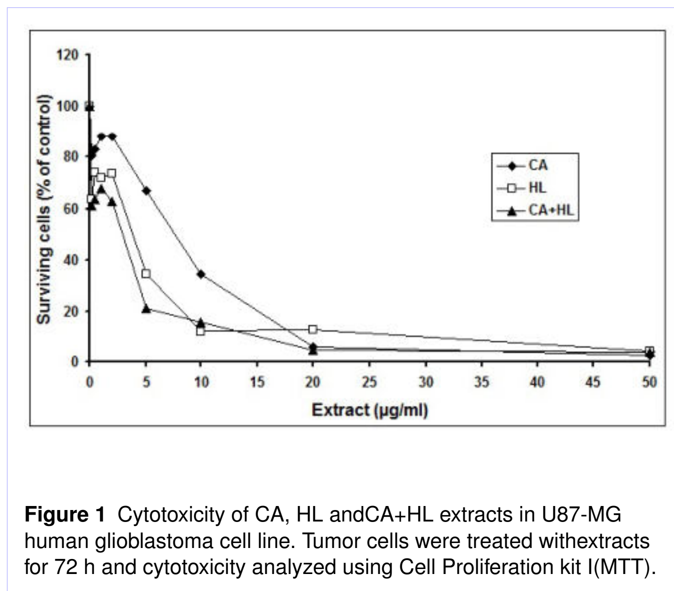

Figure 1 Cytotoxicity of CA, HL andCA+HL extracts in U87-MG human glioblastoma cell line. Tumor cells were treated withextracts for 72 h and cytotoxicity analyzed using Cell Proliferation kit I(MTT).

Table 1 Cytotoxicity ofsupercritical CO2 extract of mango ginger (CA), hops (HL) and their combinationin U-87MG human glioblastoma cell line

Treatment	IC_{50} (mg/ml)	IC_{75} (mg/ml)	IC_{90} (mg/ml)
CA	5.02 ± 0.10	12.87 ± 0.25	37.14 ± 0.19
HL	3.72 ± 0.03	22.93 ± 0.42	43.87 ± 1.04
CA + HL	2.70 ± 0.05	7.35 ± 0.21	11.64 ± 0.31

Table 2 Combination Index (CI) values between CA and HL in U-87MG cellline

Combination	CI at IC_{50}	CI at IC_{75}	CI at IC_{90}
CA+HL	0.744	0.540	0.406

<0.1 very strong synergism, 0.1-0.3 strongsynergism, 0.3-0.7 synergism, 0.8-0.9 moderateto slight synergism, 0.9-1.1 nearlyadditive, 1.1-1.45 moderate to slight antagonism, 1.45-3.3 antagonism

Cell migration

The results of cell migration analyzed by transendothelial migration assay are presented in Fig. 4. CA inhibits cell migration of tumor cells in a dose-dependent manner with approximately 62.5% and 48.5% migration at 10 m g/ml and 20 m g/ml concentrations, respectively, compared to untreated cells. The HL inhibition rate is greater than for CA, with about 47.6% and

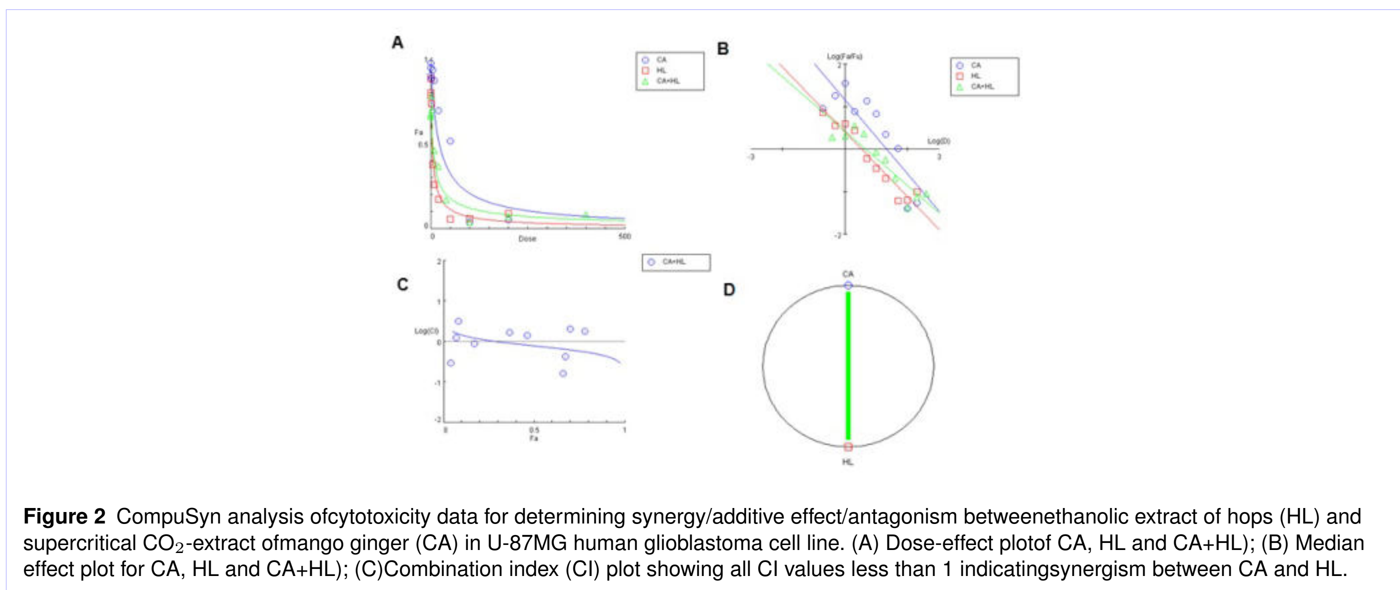

Figure 2 CompuSyn analysis ofcytotoxicity data for determining synergy/additive effect/antagonism betweenethanolic extract of hops (HL) and supercritical CO_2-extract ofmango ginger (CA) in U-87MG human glioblastoma cell line. (A) Dose-effect plotof CA, HL and CA+HL); (B) Median effect plot for CA, HL and CA+HL); (C)Combination index (CI) plot showing all CI values less than 1 indicatingsynergism between CA and HL.

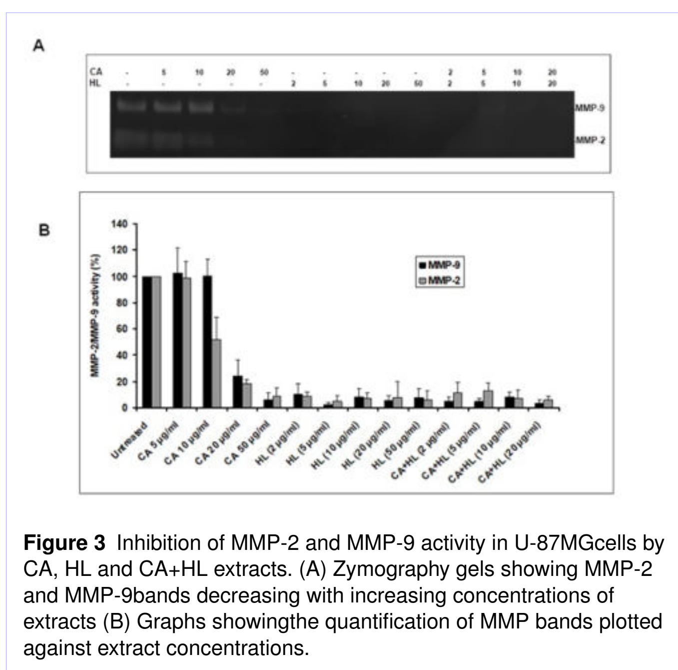

Figure 3 Inhibition of MMP-2 and MMP-9 activity in U-87MGcells by CA, HL and CA+HL extracts. (A) Zymography gels showing MMP-2 and MMP-9bands decreasing with increasing concentrations of extracts (B) Graphs showingthe quantification of MMP bands plotted against extract concentrations.

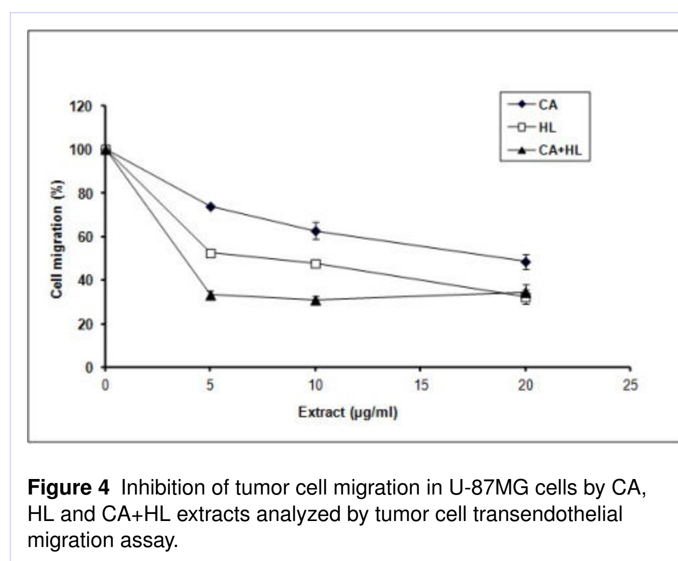

Figure 4 Inhibition of tumor cell migration in U-87MG cells by CA, HL and CA+HL extracts analyzed by tumor cell transendothelial migration assay.

32.2% migration at 10 m g/ml and 20 m g/ml concentrations. The CA+HL combination is superior to either agent alone in inhibiting tumor cell migration, with about 33.2% and 30.9% cell migration at 5 m g/ml and 10 m g/ml concentrations. At the highest dose (20 m g/ml), the CA+HL combination has comparable level of inhibition of cell migration as HL alone.

Inhibition of AKT activity

Both CA and HL significantly inhibit AKT activity significantly, although HL is more effective than CA, particularly at lower concentrations. (Fig. 5). CA inhibits AKT activity by about 70% and 80% at 20 and 50 m g/ml concentrations. HL inhibits 85% of AKT activity at 5 m g/ml and 91% at 50 m g/ml concentrations. The CA+HL combination inhibits 90% and 93% AKT activity at 20 m g/ml and 50 m g/ml concentrations, respectively.

Inhibition of cellular ATP

CA and HL inhibit ATP synthesis in U-87MG cells with CA demonstrating greater inhibition than HL (Fig. 6). While CA inhibits 88.3% ATP at 20 m g/ml concentration, HL inhibits only 51.5% ATP at that concentration. CA inhibits ATP almost completely at 50 m g/ml concentration while HL inhibits 94 % at 50 m g/ml dose. The CA+HL combination demonstrates greater

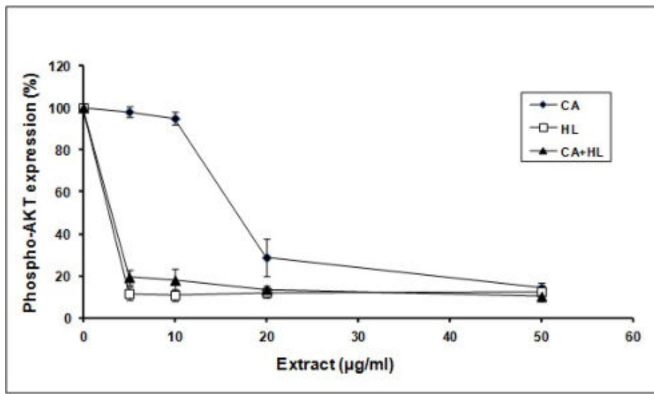

Figure 5 Inhibition of AKT phosphorylation by CA, HL andCA+HL treatment in U-87MG cell line. Phosphorylated form of AKT was analyzedusing Pathscn ELISA kit from Cell Signaling Technology

inhibition of ATP synthesis in glioblastoma cells than either extract alone. The combination treatment inhibited 95% ATP at 20 m g/ml and complete inhibition at 50 m g/ml.

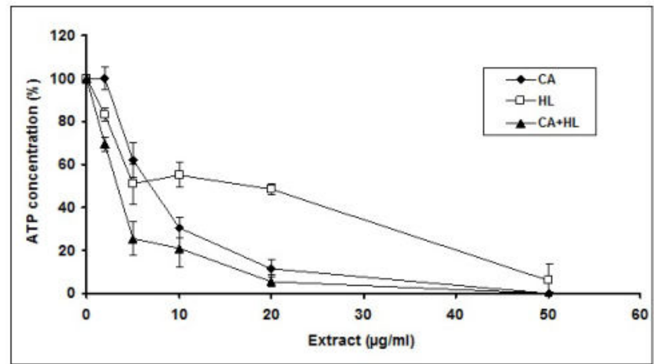

Figure 6 Inhibition of ATP synthesis by CA, HL and CA+HL treatment in U-87MG cell line. Note the increased inhibition of ATP synthesis by the combination compared to the single agents.

Inhibition of cellular lactate

Many tumor cells utilize the glycolysis pathway under aerobic conditions generating high amount of lactate in order to meet the increased demand for ATP for cell proliferation unlike normal cells. Therefore, we have analyzed whether CA and/or HL inhibits lactate production which is an indirect indicator of glycolytic activity. Both CA and HL individually and in combination inhibit lactate synthesis in glioblastoma cells (Fig. 7). The inhibition appeared to be dose-dependent, with 50 µg /ml CA showing the highest inhibitory effect (60%) on lactate production. HL reduces the cellular lactate concentration only 40 % at 50 m g/ml. The CA+HL combination demonstrates a greater effect than individual agents alone, with the lactate concentration at 38% at 20 m g/ml.

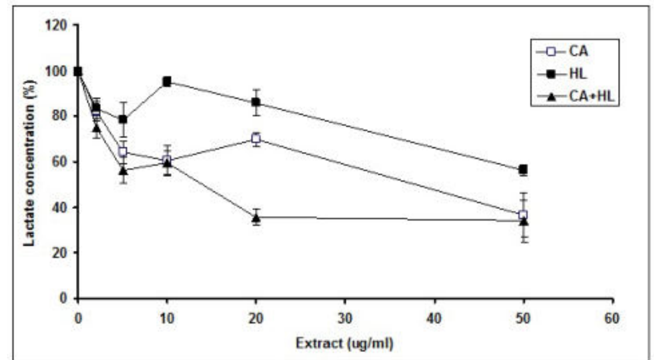

Figure 7 Inhibition of lactate synthesis by CA, HL and CA+HL treatment in U-87MG cell line. Note the increase in lactate inhibition by high concentrations of CA+HL

Expression of proteins associated with metastasis, apoptosis, inflammation and energy metabolism

The results of western blot hybridization analysis of the protein expression of genes associated with metastasis, apoptosis, inflammation and energy metabolism are presented in Fig. 8 and 10. The quantification of protein expression is presented in Fig. 9 and 11. Among the three metastasis-associated genes analyzed, CA and HL treatment appears to down regulate MMP-2 and MMP-9, the latter biomarker being down regulated more than the former. Also the tumor inhibitor of metallo proteinase protein 1 (TIMP1) is up regulated by higher doses of CA and HL as well as all the combination treatments. All the genes associated with apoptosis (mutant p53, BNIP3, BAX, Bcl-2 and caspase-3 were modulated by CA, HL and CA+HL treatments. p53, BNIP3, and Bcl-2 were down regulated while BAX and caspase-3 were up regulate d significantly. The house-keeping gene GAPDH showed no statistical difference between treatments. A dose-dependent regulation of genes associated with apoptosis (mutant p53, BNIP3, Bcl-2) and inflammation (COX-2, eNOS, HIF-1 a and mTOR) were quite apparent with CA+HL combination treatments.

Discussion

The traditional use of mango ginger and hops has suggested potential anticancer and other medicinal properties. The supercritical CO_2 extract prepared from the rhizomes of mango ginger (CA) contains 61.7% dialdehyde LDD that is shown to have cytotoxic effects against a variety of human cancer cell lines *in*

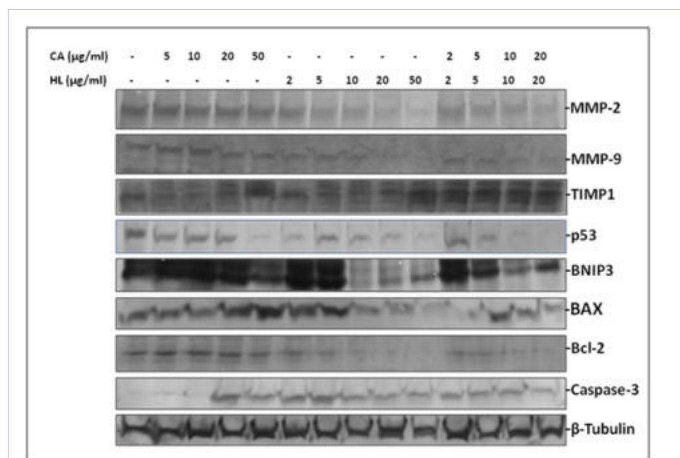

Figure 8 (A) Effect of CA, HL and CA+HL treatment on the expression of protein biomarkers associated with metastasis and apoptosis inU-87MG cell line analyzed by western blot hybridization.

vitro and *in vivo* [18, 20, 25, 26] . Previously, we have shown that CA affect a n array of cellular biochemical pathways in cancer cells inducing the tumor cell death [16–19] . CA also demonstrates synergistic cytotoxic effects with conventional cancer drugs like vinblastine, etoposide, temozolomide and irinotecan in rhabdomyosarcoma and glioblastoma cell lines [18, 19] .

The use of hops as a medicinal plant in addition to its major use in brewing industry is well known [2, 3] . The ethanolic extract of hop contains alpha-acids, beta-acids and xanthohumol which are reported to have anti-inflammatory, anticancer and estrogenic properties [15, 27–29] . The prenylflavonoids and bitter acids from hops have been shown to possess antiproliferative and anti-inflammatory effects [5, 14, 29] . In the present study, we have shown that CA and HL have synergistic effect on cytotoxicity in glioblastoma cells, as indicated by the CI values of < 1. Additionally, HL has significant antimetastatic effects because of its inhibitory effect on MMP activity. Both MMP-2 and MMP- 9 were inhibited with lower doses of HL treatment in glioblastoma cells. Furthermore, there is inhibition of tumor cell migration in the transendothelial assay by HL treatment with the CA+HL combination being more efficacious than treatment with individual agents alone. HL also inhibits AKT activity significantly in glioblastoma cells. These attributes of CA and HL indicate that these agents have potential for treatment of glioblastoma. We have previously reported on the inhibition of MMP-2 and MMP-9 expression by CA in glioblastoma cells *in vitro* [20] . Desai et al. [30] reported that META060, a modified hops extract inhibits TNF-alpha- or LPS-mediated MMP-9 levels and enzyme activity. Furthermore, xanthohumol, a flavonoid from hop s inhibits MMP activity [31] . Similarly, myricetin and quercetin, two flavonoids present in hops extract have been shown to inh ibit MMP-2 and MMP-9 expression, indicating the anti-angiogenic properties of these compounds [32–34] .

The role of AKT on the survival and growth of cancer cells is well-established. Many human cancers including glioblastomas exhibit frequent activation of Akt that correlate s with advanced disease and /or poor prognosis [35] . The penetration of circulating tumor cells into the endothelium is a crucial step in tumor metastasis. We have previously shown that CA treatment significantly inhibits cell migration in glioblastoma cells in a dose-dependent manner [18] . Cell migration is one of the pre-requisites of metastasis and glioblsatomas are one of the highly metastatic malignancies in humans. Cancer invasion and metastasis involve the degradation of extracellular matrix involving matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9 [36–38] . In the present investigation, the CA+HL combination treatment inhibited the tumor cell migration more than the individual agents. Also HL and CA+HL have inhibited the both expression and activities of MMP-2 and MMP-9 and up regulated the expression of TIMP1 protein expression. Further, HL (in addition to CA) and the CA+HL combination down regulate the phosphorylation of AKT. Many tumor cells utilize aerobic glycolysis with reduced oxidative phosphorylation for glucose metabolism, a phenomenon referred to as the Warburg effect and glioblastomas has been shown to have a preferential metabolism of glucose through aerobic glycolysis. Recently we showed that the combination of CA with glycolytic inhibitors such as 2-Deoxy-D-glucose (2-DG) and S odium oxamate (SO) inhibit growth, proliferation and migration of glioblastoma cells [24] . CA+HL combination in the present study has more inhibitory effect on lactate and ATP synthesis indicating the superior effect on inhibition of glycolytic pathway than CA or HL alone. Such increased inhibition of glycolysis for drug combinations compared to single agents has been reported in breast cancer cells [39, 40] and glioblastoma cells [24] .

Gene expression studies in the current study indicated that CA , HL and the CA+HL combination up regulate the expression of pro-apoptotic genes (BAX and caspase 3) and down regulate anti-apoptotic genes (mutant p53, BNIP3 and Bcl-2). Furthermore, the combination down regulates markers associated with inflammation and energy metabolism such as COX-2, iNOS, AMPK a, HIF-1 a and mTOR proteins. In a recent study, we reported that CA could down regulate these markers and the fact that the CA+HL combination is synergistic suggests that evaluation of this combination for therapeutic management of glioblastoma is warranted. In conclusion, the results from this investigation indicate the anticancer and antimetastatic properties of CA and HL and the feasibility of combining these two

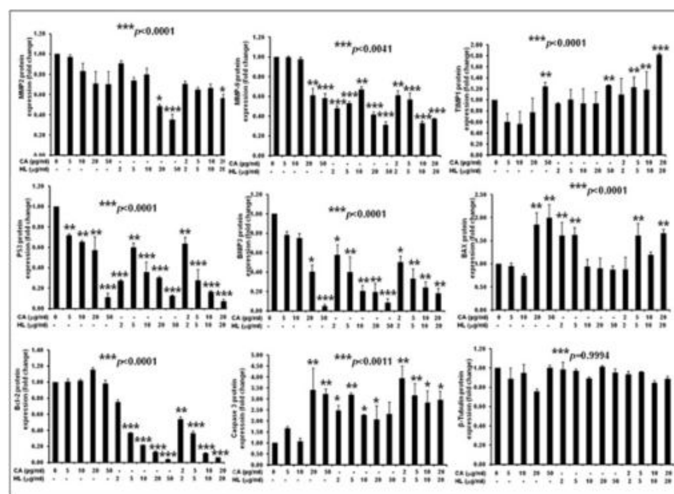

Figure 9 Quantification of expression of protein markers(western blots) associated with metastasis and apoptosis in U-87MG cell line. The relative expression levels (fold level) are plotted against extract concentrations. The significant difference between treatments was estimated by1-way analysis of variance with Dunnett's multiple comparison test (GraphPadPrism software, La Jolla, CA) (*p< 0.05, **p <0.01, ***p < 0.001).

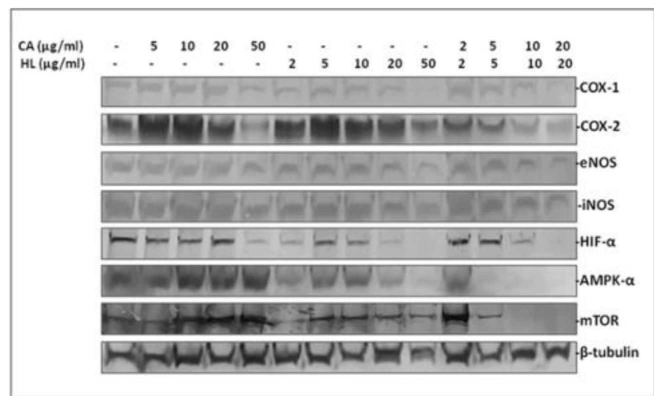

Figure 10 Effect of CA, HL and CA+HL on the expression of protein markers associated with inflammation and energy metabolism in U-87MG cell line analyzed by western blot hybridization.

biological extracts against glioblastoma, the validity of which has to be analyzed in animal models and human trials.

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.

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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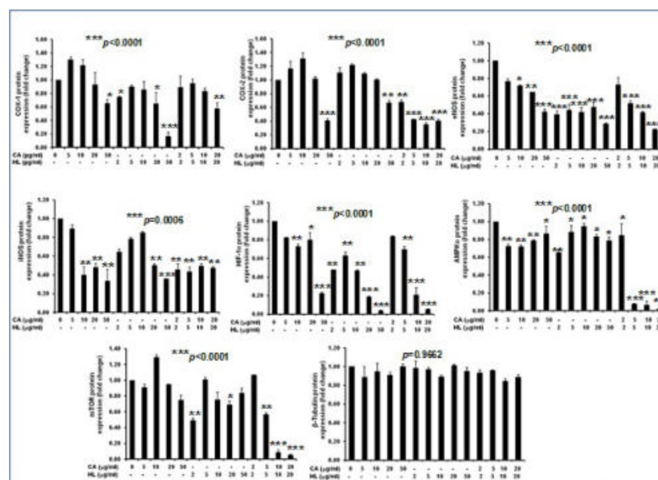

Figure 11 Qantification of protein expression (westernblots) associated with inflammation and energy metabolism in U-87MG cell line.The relative expression levels (fold level) are plotted against extractconcentrations. The significant difference between treatments was estimated by 1-wayanalysis of variance with Dunnett's multiple comparison test (GraphPad Prismsoftware, La Jolla, CA) (*p <0.05, **p < 0.01, ***p < 0.001).

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