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In Vivo Antitumor Effect of Supercritical CO_2 Extract of Mango Ginger (*Curcuma amada* Roxb) in U-87MG Human Glioblastoma Nude Mice Xenografts

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Abstract

Glioblastoma multiforme (GBM) is one the most aggressive and lethal human neoplasms with poor prognosis and very limited positive treatment options. The antitumor effect of supercritical CO_2 extract of mango ginger (*Curcuma amada* Roxb) (CA) with and without irinotecan (IR) was analyzed in U-87MG human glioblastoma multiforme (GBM) cells *in vitro* and in nude mice xenografts. CA is highly cytotoxic to GBM cells and is synergistic with IR as indicated by the combination index values of <1 in the CompuSyn analysis. CA inhibits tumor growth rate in GBM xenografts, the inhibition rate being higher than in IR treated group. GBM xenograft mice treated with IR + CA combination showed almost complete inhibition of tumor growth rate. Gene expression analysis of xenograft tumors indicated that IR + CA treatment significantly downregulated anti-apoptotic (Bcl-2 and mutant p53), inflammation-associated (COX-2) and cell division–associated (CCNB2) genes and upregulated pro-apoptotic genes (p21 and caspase-3). These results confirmed the therapeutic efficiency of IR + CA combination against GBM and the need for further clinical investigations.

Keywords

mango ginger, *Curcuma amada*, glioblastoma, xenografts, therapeutic ratio, gene expression

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Glioblastoma multiforme (GBM) is one of the most aggressive and lethal forms of human malignancies, with a median survival of only 12 to 15 months in patients undergoing current standard treatment involving surgery, chemotherapy, and radiation therapy.[1,2] In general, 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 cancer cells.[3,4] Despite the introduction of combination chemoradiotherapy with temozolomide,[5] treatment options remain limited for gliomas. Because of these dismal statistics, it is quite imperative to identify novel therapeutic agents either as single or adjuvant agents for gliomas. Interestingly, surveys have shown that a majority (77%) of the high-grade glioma patients use complementary and alternative medicine adjuvantly.[6,7] It is also reported that for any agent to be efficacious and successful for brain tumor therapy, the molecule must at a minimum be lipid soluble so as to diffuse across the blood-brain barrier.[8]

Mango ginger (*Curcuma amada* Roxb) is a plant of the Zingiberaceae family that is closely related to turmeric (*Curcuma longa* L). *C amada*, used for culinary purposes and traditional medicine application in India and other parts of Asia, is more commonly known as mango ginger because the rhizomes (roots) are very similar to ginger and have a profound raw mango taste. The supercritical CO_2 extract of *Curcuma amada* (CA) has shown significantly higher in vitro anticancer activity than curcumin and turmeric (*C longa*) in brain tumor cells.[9,10] CA combined with other cancer drugs like vinblastine, temozolomide, etoposide, and cyclophosphamide also demonstrated synergistic effects for *in vitro* cytotoxicity in cancer cell lines.[9-11] Among the important effects of CA demonstrated in glioblastoma cells, are the downregulation of the gene expression associated with inflammation

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such as COX-2 and NF-κB, induction of apoptosis in a dose-dependent manner and downregulation of genes associated with anti-apoptosis, cell proliferation, telomerase activity, oncogenesis, and drug resistance. In this investigation, we have analyzed the antitumor effect of CA and irinotecan (one of the popular neoplastic drugs used for the chemotherapy of brain tumor patients)[12] individually and in combination in human glioblastoma cells *in vitro* and in nude mice xenografts in vivo.

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% fetal bovine serum and antibiotics in a humidified 5% CO_2 incubator maintained at 37°C.

Drugs

Supercritical CO_2 extract of mango ginger (CA) was prepared by Flavex Naturextrakte GmbH, Rehlingen, Germany. The supercritical CO_2 extraction process is designed to extract primarily nonpolar molecules yielding a uniform product without any residue of organic solvents unlike other solvent extraction methods and with a high level of batch to batch consistency. The chemical fingerprint details of CA have been described in our earlier publication.[10] Irinotecan hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO) for the investigation.

Cytotoxicity

Glioblastoma (U-87MG) cells were treated with increasing concentrations of IR, CA, and combinations of IR and CA (0-200 μg/mL) for 72 hours in 96-well plates. In the combination treatment, both CA and IR were combined in equal amounts at increasing concentrations from 0.2 to 200 μg/mL doses for comparison with single agents. MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay performed with the Cell Proliferation Kit I (Roche Biochemicals, Indianapolis, IN) was used to analyze cytotoxicity of individual drug/extract and their combination. The experiment was repeated 4 times with 3 replications for each treatment and the IC_{50} (50% inhibitory concentration), IC_{75}, and IC_{90} vales were calculated.[9,10]

CompuSyn Analysis

To determine the synergistic/additive/antagonistic effect between IR and CA, cytotoxicity data were analyzed further using CompuSyn software (CompuSyn Inc, Paramus, NJ). This program is based on Chou and Talalay's multiple drug effect equations[13] and it defines synergism as a more-than expected additive effect and antagonism as a less-than expected additive effect. 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.[13,14]

Table 1. Cytotoxicity of CA, IR, and IR + CA in U-87MG Cells.

Treatment	IC_{50} (μg/mL)	IC_{75} (μg/mL)	IC_{90} (μg/mL)
CA	4.93 ± 0.81	13.47 ± 0.86	23.10 ± 1.13
IR	3.95 ± 0.11	9.71 ± 0.85	15.15 ± 1.85
IR + CA	3.23 ± 0.15	7.83 ± 0.69	12.10 ± 1.06

Abbreviations: CA, supercritical extract of *Curcuma amada*; IC, inhibitory concentration; IR, irinotecan.

Xenograft Studies

Male athymic nude (n/nu) mice of *NCRNU* strain (4 weeks old weighing 18-22 g) were purchased from Taconic Labs (German Town, NY). Human glioblastoma U-87MG cells (10^7 cells/site) grown in tissue culture were suspended in 0.1 mL of Hank's balanced salt solution and were injected subcutaneously into the left and right flank of the nude mice with a 21-gauge needle. When the tumors measured 0.5 cm^3, therapy was started with 20 mice in each group. Treatment groups included control (saline), IR (50 mg/kg), CA (50 mg/kg), and IR (50 mg/kg) + CA (50 mg/kg). The IR and CA doses would be equivalent approximately to the *in vitro* dose of 50 μg/ml. IR was dissolved in phosphate buffered saline and administered intraperitoneally twice per week at 4-day intervals for a total of 10 injections starting on the first day of tumor measurement and treatment. CA was dissolved in olive oil and administered by oral gavage twice per week at 4-day intervals for a total of 20 treatments. Tumor size (length × width), body weight, and survival data were collected twice a week. Tumor measurements were recorded using a Vernier caliper, and tumor volume was calculated.[15] Tumor growth rate, tumor growth inhibition rate, and therapeutic ratio were calculated according to the formula described earlier.[10,15,16] Tumor growth rate data in different treatment groups were analyzed by Student's *t* test in order to estimate the significant difference between them. Survival data were analyzed with Kaplan-Meier statistics using Graphpad Prism software (La Jolla, CA) and significant differences among treatment groups were calculated.

Gene Expression Analysis in Tumor Tissues

Tumors were dissected from 3 animals at the end of the study and stored in TriZol reagent (Life Technologies, Inc, Grand Island, NY) for RNA extraction. RNA was extracted from tumor tissues using TriZol reagent according to manufacturer's protocol. The mRNA expression of genes associated with apoptosis(APO-BEC, Bax, Bcl-2, Bax/Bcl-2 ratio, BNIP3, p53, p21, STAT3, and caspase-3) as well as cell proliferation and angiogenesis (MMP2, MMP9, TIMP1, TIMP2, COX-2, CCNB2, VEGF, and N-myc) were analyzed by reverse transcriptase–polymerase chain reaction.[9,10] The expression of housekeeping gene β-actin was used as control. The relative expression of genes was quantified using gel pictures by the UNSCAN-IT gel software (Silk Scientific, Inc, Orem, UT). The fold-level changes in the expression of various genes were plotted against treatment groups.

Statistical Analysis

Mean IC values and standard deviation estimates were calculated using Microsoft Excel software. The fraction of surviving cells at each concentration of drugs/combinations was used for the analysis of synergy/additivity/antagonism between IR and CA by the CompuSyn software (ComboSyn, Inc, Paramus, NJ).

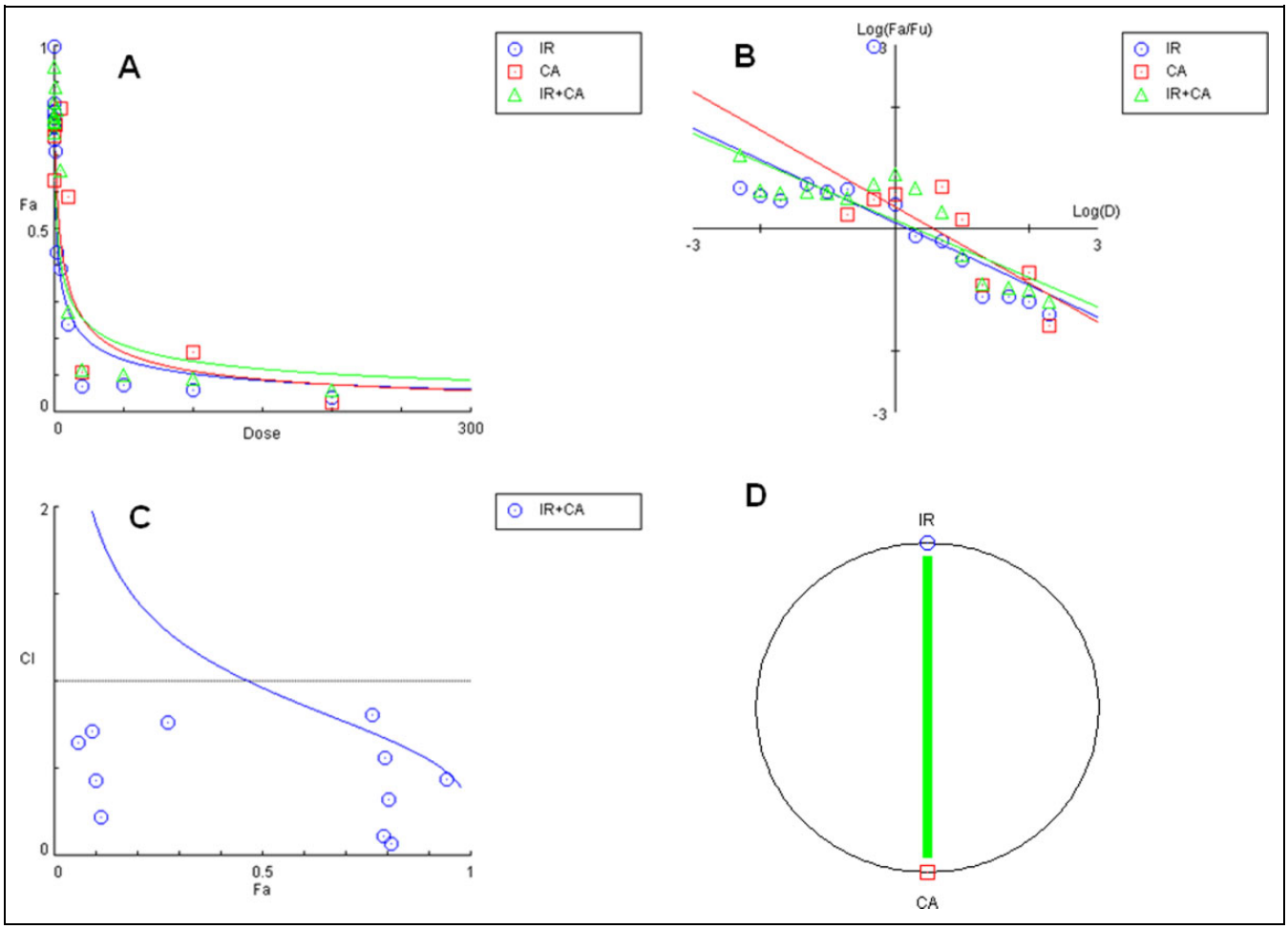


Figure 1. CompuSyn analysis of cytotoxicity data for determining synergy/additivity/antagonism between supercritical CO_2 extract of *Curcuma amada* (CA) and irinotecan (IR) in U-87MG glioblastoma cell line. (A) Dose-effect plot of IR, CA, and IR + CA. (B) Medium effect plot for IR, CA and IR + CA. (C) Combination index (CI) plot showing all CI values less than 1 indicating synergism between IR and CA. (D) Polygonogram with green thick line indicating the synergistic effect between IR and CA.

The relative mRNA expression (average pixel units) for different CA concentrations was statistically analyzed by 1-way analysis of variance and the treatments were compared using Dunnett's multiple comparison test (GraphPad Prism software, La Jolla, CA).

Results

Cytotoxicity

CA demonstrated significant cytotoxicity in U-87MG cells with IC_{50}, IC_{75}, and IC_{90} values of 4.93, 13.47, and 23.10 µg/mL, respectively (Table 1). IR, an effective drug against brain neoplasms, yielded IC_{50}, IC_{75}, and IC_{90} values of 3.95, 9.71, and 15.15 µg/mL, respectively. When IR and CA were combined, there was improvement in cytotoxicity with 3.23, 7.83, and 12.10 µg/mL at IC_{50}, IC_{75}, and IC_{90} levels.

Synergy Between CA and IR

CompuSyn analysis to determine the synergy/additivity/antagonism between CA and IR is given in Figure 1. The dose-effect (A) median-effect plots (B), CI/Fa plot (C), and the polygonogram indicate the synergy between CA and IR. CI plot shows all the points falling below 1. The combination index (CI) at IC_{50}, IC_{75}, and IC_{90} are 0.963, 0.719, and 0.550, respectively, indicating the synergy between CA and IR at all levels.

Inhibition of Tumor Growth

The average tumor growth rates of U-87MG glioblastoma in nude mice xenografts of different treatment groups are presented in Figure 2. Generally, tumors grew in a similar rate in all groups up to the 16th day of the start of treatment, thereafter the IR + CA combination group showed continued significant inhibition of tumor growth compared to all other groups. The inhibition of tumor growth in IR and CA groups became obvious after 30 days of treatment compared with the control group. Again from 40th day of treatment onward, the inhibition by CA treatment appeared to be significantly better than that of IR. Tumor growth rate increased steadily for the

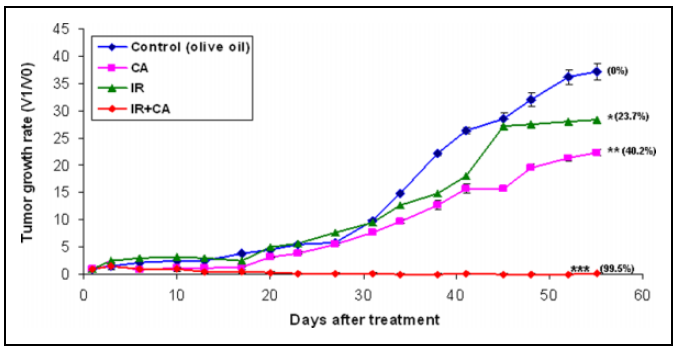


Figure 2. Inhibition of tumor growth rate in U-87MG nude mice xenografts treated with olive oil (control), 50 mg/kg supercritical CO_2 extract of mango ginger (CA) in olive oil, irinotecan (IR) 50 mg/kg, and IR 50 mg/kg + CA 50 mg/kg combination. Olive oil (control) and CA were given through oral gavage and IR was injected intraperitoneally. The treatment details are given in Materials and Methods section. The reduction in the tumor volume (percentage) for each treatment group calculated on the basis of the control group is presented in the figure.

olive oil–fed (control) group reaching about 37.23 times on 55th day compared with original size of 1 on the first day. Both CA and IR treatments group showed a significant decrease in the tumor growth rate. While IR treatment reduced the tumor volume by 23.7% (compared with control), CA treatment reduced the volume by 40.2%. On the other hand, IR + CA treatment reduced the tumor volume by 99.5% (Figure 2). On the 55th day, the average tumor growth rate was 28.35 times of the original size for IR treatment ($P < .05$). The growth rate in the CA treated group demonstrated a more significantly reduced growth rate with the average tumor growth rate of 22.33 times the size on day 1 ($P < .01$). The IR + CA combination treatment showed dramatic inhibition of tumor growth, as the growth rate was only 0.18 ($P < .001$). The therapeutic ratio given in Table 2 indicates the efficiency of IR and CA in nude mice xenografts. CA alone has a therapeutic ratio of 40.03 as compared with 23.85 for IR. The IR + CA group has a therapeutic ratio of 99.52. Therefore, CA either alone or in combination with IR is significantly more efficacious than IR in terms of therapeutic efficiency. Furthermore, the combination showed significantly better results than single agents.

Survival Analysis

The results of Kaplan-Meier survival analysis is presented in Figure 3 and Table 2, which showed that both CA and IR + CA treatment groups survived longer than IR and control treatments ($P < .05$). IR group was slightly better survival than control group.

Gene Expression

The mRNA expression of genes associated with apoptosis, inflammation, angiogenesis, and cell proliferation are given

Table 2. Therapeutic Ratio and Survival of U-87MG Xenografts.

Treatment	Therapeutic Ratio	Survival (%)
Control (olive oil)	0	30.01
CA	40.03	40.00
IR	23.85	27.27
CA + IR	99.52	60.33

Abbreviations: CA, supercritical extract of *Curcuma amada*; IR, irinotecan.

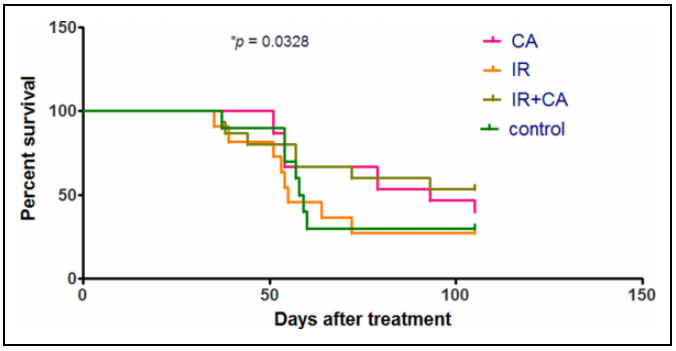


Figure 3. Kaplan-Meier survival curve analysis of U-87MG nude mice xenografts treated with control (olive oil), CA 50 mg/kg, IR 50 mg/kg, and IR 50 mg/kg + CA 50 mg/kg. Significant differences between treatment groups are indicated by the *P* value (*$P < .05$).

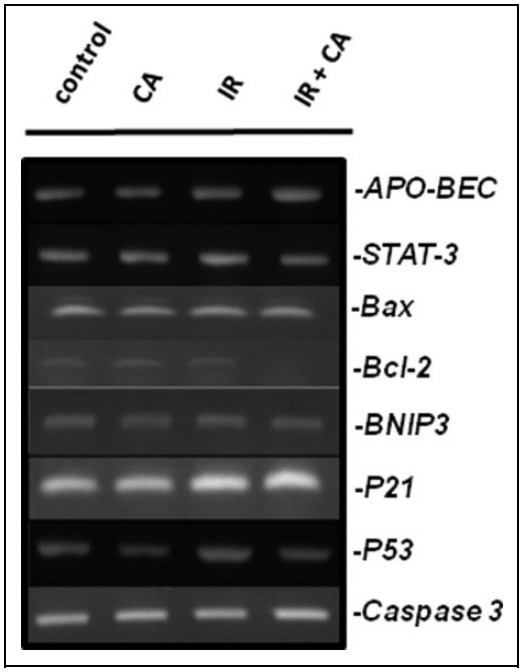


Figure 4. Gene expression analysis of apoptotic genes (*APO-BEC*, *STAT3*, *Bax*, *Bcl-2*, *BNIP3*, *P53*, *p21*, and *caspase-3*) by reverse transcriptase–polymerase chain reaction assay in U-87MG xenograft tumors established in nude mice that has been treated with control, CA, IR, and IR + CA treatments.

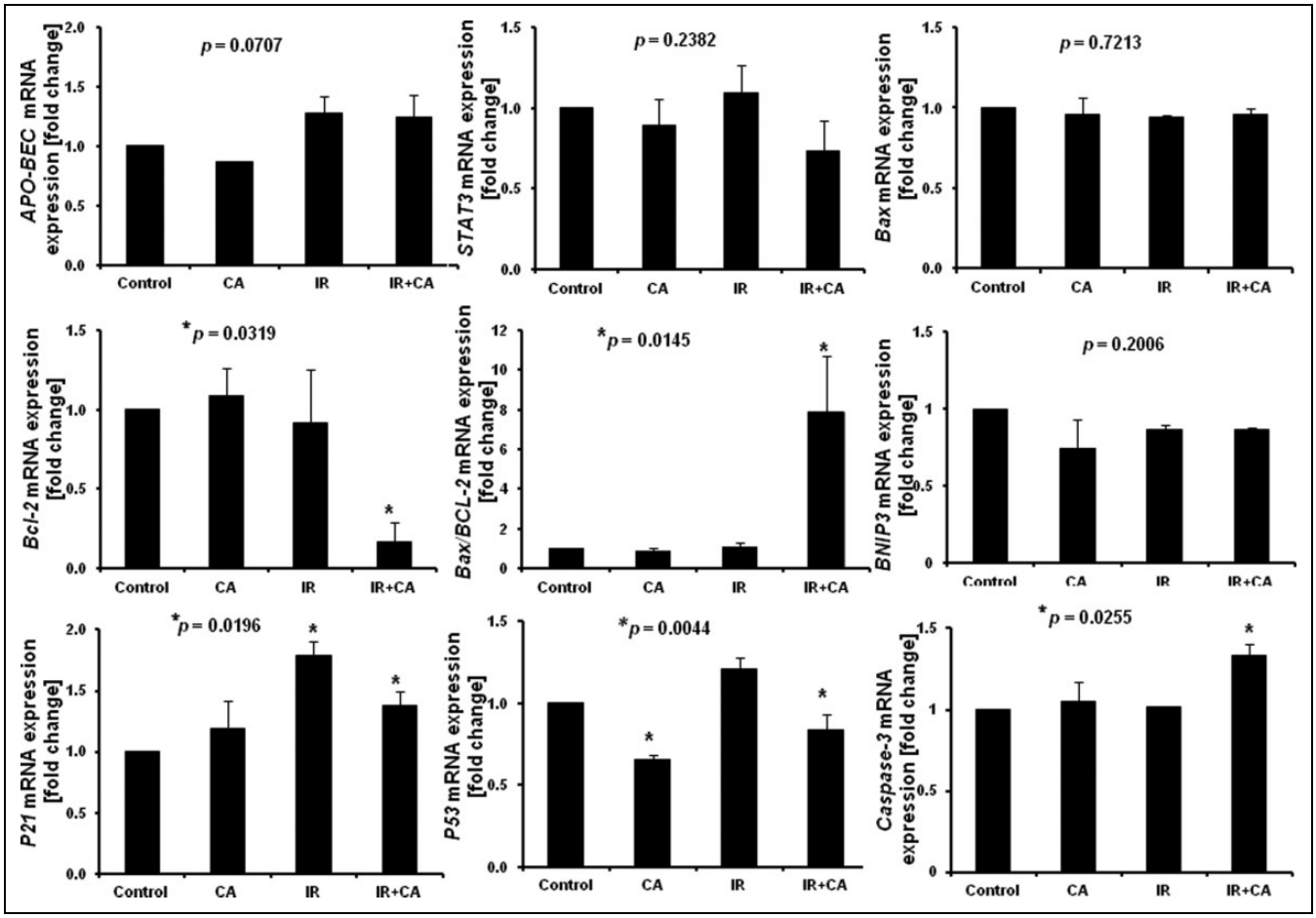


Figure 5. Quantification of expression of apoptotic genes by UNSCAN-IT gel software. The relative changes in the expression of genes (fold change) were plotted against treatment groups. The significant difference between treatment groups is analyzed by 1-way ANOVA with Dunnett's multiple comparison test (GraphPad Prism software, La Jolla, CA) (*$p < 0.05$).

in Figures 4 and 6 and the quantified values are presented in Figures 5 and 7. Treatment of glioblastoma xenografts with IR + CA combination significantly downregulated *Bcl-2* and mutant *p53*, and increased *Bax/Bcl-2* ratio. The combination also upregulated *p21* and *caspase-3* genes, probably accounting for the prominent cure rate observed in xenografts. The combination also showed significant downregulation of VEGF and N-myc mRNA expression. While CA and IR + CA treatment groups showed significant downregulation of inflammation-associated COX-2 mRNA expression, both IR and IR + CA treated xenografts showed downregulation of CCNB2 mRNA, a checkpoint gene involved in cell division of tumor cells.

Discussion

Glioblastoma multiforme (GBM) is the most common intracranial cancer of astrocytic origin.[17] Improving therapy for patients with GBM is one of the greatest challenges in oncology. Despite the use of multimodal therapy (surgery and radiochemotherapy with temozolomide) the prognosis of patients with this highly aggressive malignant neoplasm remains poor with a median survival time of 14.6 months.[5] The identification and development of novel therapeutic agents and strategies therefore remains an important priority for treatment of this disease. Targeted therapies, as single agents, have failed to offer significantly enhanced survival benefit, despite objective initial responses.[18] Glioblastoma heterogeneity and complex molecular underpinnings likely contribute to the resistance to conventional therapeutic modalities. Therefore, glioblastoma might be more efficiently treated by targeting multiple signaling pathways or different tumor features (proliferation, resistance to apoptosis, angiogenesis, and invasion) simultaneously.[19]

In our investigations *in vitro*, we have established that CA has anticancer potential against human glioblastoma cells.[9,11] CA showed significantly higher cytotoxicity than temozolomide, etoposide, curcumin, and turmeric force. The inhibitory concentration values of CA for normal embryonic mouse hypothalamus cell line (mHypoE-N1) is significantly higher than glioblastoma cell line, indicating the specificity of CA against brain tumor cells. CompuSyn analysis indicated that CA acts synergistically with temozolomide and etoposide for the cytotoxicity with combination index values of <1. CA

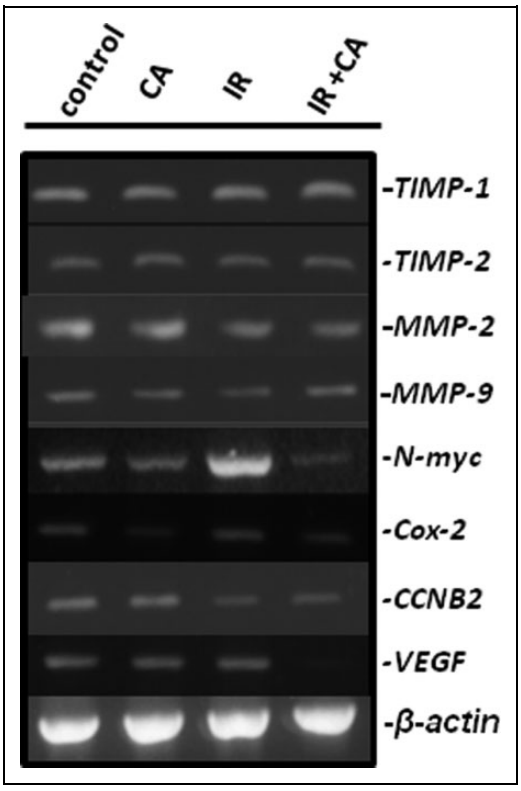


Figure 6. Analysis of mRNA expression of genes associated with cell invasion, angiogenesis and inflammation (*TIMP1*, *TIMP2*, *MMP2*, *MMP-9*, *N-myc*, *COX-2*, *CCNB2*, and *VEGF*) along with β-actin (control) gene by RT-PCR assay in U-87MG xenograft tumors established in nude mice. Xenografts were treated with olive oil (control), CA, IR, and IR + CA. CA, supercritical CO_2 extract of *Curcuma amada*; IR, irinotecan.

treatment of glioblastoma cells induced apoptosis in a dose-dependent manner and downregulates genes associated with anti-apoptosis, cell proliferation, telomerase activity, oncogenesis, and drug resistance.[9] Recently, we reported that CA inhibits AKT and AMPKα phosphorylation significantly in a dose-dependent manner. The cell migration which is necessary for invasion and metastasis was also inhibited by CA treatment. Analysis of mRNA and protein expression of genes associated with apoptosis, cell proliferation and angiogenesis showed that CA modulates expression of genes associated with apoptosis (*Bax*, *Bcl-2*, *Bcl-X*, *BNIP3*, *caspase-3*, *mutant p53*, and *p21*), cell proliferation (*Ki67*), and angiogenesis (*VEGF*) in vitro. Additionally, *HSP90* and *AMPKα* genes interacting with the AKT signaling pathway were also downregulated by CA treatment. These results indicate some of the important molecular targets and mechanisms underlying the anticancer effect of CA in human glioblastoma cells.

In the present investigation, CA was highly synergistic with IR in the glioblastoma cell line (U-87MG). Consequently, the antitumor effects of these 2 agents were tested both individually and in combination with U-87MG xenograft model developed in athymic nude mice. The results show the anticancer effect of CA and IR both individually and in combination. Treatment of xenograft mice with IR, CA and IR+CA inhibited tumor growth significantly as compared with olive oil–fed control group. The inhibitory effect was greater for CA than for IR. However, the IR+CA group yielded significantly greater inhibition of tumor growth than either the CA or IR groups and also yielded an almost complete shrinkage of tumor in this group. The survival data analysis also show longer survival in IR, CA, and IR + CA treated xenografts compared with the control group with the longest survival in IR + CA group, followed by CA and IR groups in order. The therapeutic ratio of CA either alone or in combination with IR is significantly higher than IR alone in glioblastoma xenografts indicating better efficacy than IR. While CA reduced the tumor volume by 40.2%, IR + CA combination completely inhibited the tumor growth with a 99.5% reduction in tumor volume. Therefore, the combination has significantly better therapeutic effect than either agent alone. These *in vivo* results are in conformity with the synergy observed with CompuSyn analysis of *in vitro* data. In a recent article, we reported that CA is a potent AKT inhibitor in glioblastoma cell line, U-87MG.[11] CA inhibits AKT and AMPKα phosphorylation significantly in a dose-dependent manner. Recently, several inhibitors of the P13K-AKT signaling pathway are being evaluated in clinical trials against GBMs.[20,21] The interesting aspect of CA is that it inhibits several targets promoting initiation, growth and metastasis of GBMs, which suggests that CA may have applications as an adjuvant for GBM therapy. Our earlier *in vitro* studies have also shown that CA inhibits mTOR and VEGF expression contributing to its multitarget use against GBMs.[9] Xenograft studies in the present investigation also showed that IR + CA combination downregulates anti-apoptotic genes, including *Bcl-2* and mutant *p53*, and upregulates *Bax/Bcl-2* ratio, *p21*, and *caspase-3*, consistent with our observation of the induction of apoptosis in our earlier *in vitro* studies.[9,11] IR + CA treatment of xenografts also downregulates *VEGF* and *N-myc* mRNA expression, which may inhibit tumor growth and angiogenesis. In addition, the inflammation-associated *COX-2* gene and the cell division-associated *CCNB2* gene are downregulated with IR + CA treatments. These markers were also downregulated in our previous *in vitro* investigations with U-87MG human glioblastoma cells.[9] In summary, our xenograft studies confirmed the therapeutic efficiency of IR + CA combination against human glioblastoma cells *in vivo* and these results support the value for a clinical trial with CA as an adjuvant agent in the treatment of patients with GBM or other high-grade gliomas, for which very few successful therapeutic options are currently available.

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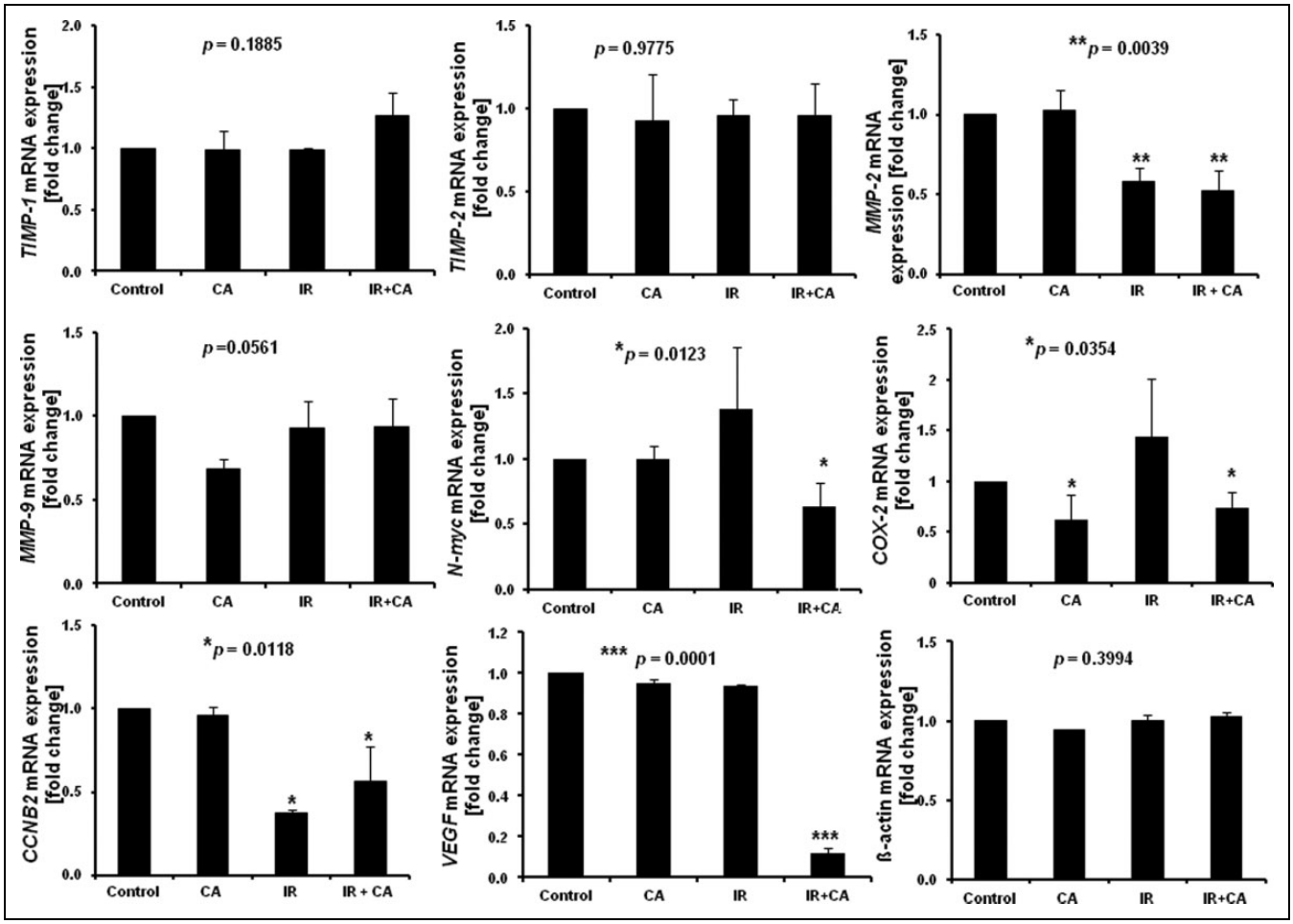


Figure 7. Quantification of expression of genes associated the cell invasion, inflammation, and angiogenesis by UNSCAN-IT gel software. The relative changes in the expression of genes (fold change) were plotted against treatment groups. The significant difference between treatment groups is analyzed by 1-way analysis of variance with Dunnett's multiple comparison test (GraphPad Prism, La Jolla, CA). (*$P < .05$, ***$P < .001$).

Author Contributions

CR and SJM were responsible for study design, experimentation, and article preparation. GMP and AMP were actively involved in the experimentation and article preparation. K-WQ formulated and prepared the supercritical CO_2 extract of mango ginger for the investigation and performed chemical analysis. All authors have read and approved the article.

Declaration of Conflicting Interests

The authors declared 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 Adriana M. Prado are also employees of Dharma Biomedical LLC.

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Ethical Approval

To perform this investigation, necessary approval for undertaking the nude mice xenograft study was obtained from the Institutional Animal Care and Use (IACUC) committee of Nicklaus Children's Hospital, Miami, Florida, USA; study was performed adhering to the IACUC rules and regulations. This investigation does not involve any human subjects.

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