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**SYNERGISTIC ANTITUMOR EFFECT OF ASCORBIC ACID, LYSINE,
PROLINE, AND EPIGALLOCATECHIN GALLATE ON HUMAN
FIBROSARCOMA CELLS HT-1080**

M.Waheed Roomi, Vadim Ivanov, Aleksandra Niedzwiecki, and Matthias Rath

Matthias Rath, Inc., R&D, Cancer Division, Santa Clara, CA 95050

Corresponding Author – Alexandra Niedzwiecki, Ph.D., Matthias Rath, Inc., 1260 Memorex Drive, Santa Clara, CA, 94050 USA. Phone – 408-567-5564, Fax – 408-986-9403. Email – a.niedz@drrath.com.

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Synopsis

Introduction: Matrix metalloproteinases (MMPs) have received much attention in recent years for their role in various malignancies, and have been implicated in tumor invasion, metastasis and angiogenesis. Certain MMPs, such as MMP-2 and MMP-9, have shown a special propensity for promoting cancer. Development of MMP inhibitors has been a recent approach to controlling cancer and blocking metastasis. Objective: We investigated the effect of EGCG individually and in combination with lysine, proline, and ascorbic acid (LPA), *in vitro*, on human fibrosarcoma cells HT-1080, by measuring: cytotoxicity, modulation of MMP-2 and MMP-9, and invasive potential. Fibrosarcoma cell line was chosen for this study as it expresses both MMP-2 and MMP-9. Methods: Cytotoxicity was evaluated based on cell proliferation by MTT assay and MMP expression in condition media by gelatinase zymography. Invasion was evaluated through Matrigel. Results: Treatment of fibrosarcoma cells with EGCG independently showed dose-dependent cytotoxicity that was enhanced when EGCG was combined with LPA,

with a maximum toxic effect of 45% at 50 µg/ml EGCG + LPA. Zymography showed dose dependent inhibition of MMP-2 and MMP-9 expression by EGCG, also enhanced at each concentration when combined with LPA, with virtual total inhibition of MMP-2 at LPA + EGCG 20 µg/ml and MMP-9 at LPA + EGCG 50 µg/ml concentration. The invasion of fibrosarcoma cells through Matrigel was significantly reduced (63%) with LPA + EGCG 20 µg/ml and totally inhibited with LPA + EGCG 50 µg/ml. Conclusion: Our results suggest that the synergistic effect of lysine, proline, and EGCG, is an effective, yet safe agent for adjunctive therapeutic use in the treatment of fibrosarcoma, by inhibiting cell proliferation, MMP expression, and matrigel invasion.

Introduction

Tumor metastasis is a multi-step process involving biochemical events that leads to destruction of the basement membrane and connective tissues by invasion of malignant cells. In early stages of invasion, the ECM exhibits increased permeability to cell movement, due either to decreased synthesis of matrix components or to ECM degradation by tumor cell-associated proteases. Metastatic cells secrete high levels of MMPs, which can degrade ECM components. An increased expression of MMPs in tumor cells has been correlated with their metastatic potential [DeClerck, Imren *et al.* (1997)]. Among the MMP family, MMP-2 (gelatinase A, 72-kDa) and MMP-9 (gelatinase B, 92-kDa) have been correlated with aggressiveness in human cancer and seem to play an important role in tumor invasion and metastasis [Chambers and Matrisian (1997)]. These two type IV collagenases are the MMPs predominately released by most epithelial and endothelial cells. MMPs also play an important role in tissue remodeling, wound healing, angiogenesis and heart disease.

We have developed a natural, effective approach to inhibiting cancer cell growth and spread, using selected nutrients, such as lysine, proline, ascorbic acid, and EGCG. These nutrients, used in synergy, can enhance cellular mechanisms that promote natural encapsulation of cancer tumors, including inhibition of MMPs and strengthening of connective tissue surrounding cancer cells. Rath and Pauling suggested that nutrients, such as the amino acid lysine and ascorbic acid can act as natural inhibitors of ECM proteolysis [Rath and Pauling (1992)]. Lysine and its derivatives act as inhibitors of

plasmin, and thereby can modulate plasmin-induced MMP activation cascade [Rath and Pauling (1992)]. In addition, lysine, proline and vitamin C are essential nutrients for optimum synthesis and structure of collagen and the stability of connective tissue [Murad, Grove *et al.* (1981)]. Ascorbic acid is a potent scavenger of free radicals and thus protects cells from damage [Dumitresca, Belgun *et al.* (1993)]. EGCG, derived from green tea, has strong antioxidant and anticarcinogenic properties [Mukhtar and Ahmed (2000)]. It is postulated that the combination of these nutrients exert synergistic, anticancer activity at the cellular level and, as such, they have the potential to modulate tumor growth and expansion.

In the present study we have investigated the effect of EGCG alone and EGCG with lysine, proline and ascorbic acid on proliferation, MMP expression (MMP-2 and MMP-9), and invasion potential through Matrigel, all important parameters for cancer prevention, using fibrosarcoma cell line HT-1080. This cancer cell line expresses both MMP-2 and MMP-9, both of which are involved in cancer progression and metastasis.

Materials and Method

Cell Culture

Human fibrosarcoma cells HT-1080 were obtained from ATCC (American Type Culture Collection, Rockville, MD) and grown in MEM medium supplemented with 10% fetal bovine serum, penicillin G sodium (100 U/ml), streptomycin (100 µg/ml), and amphotericin (0.25 µg/ml) in 24-well tissue culture plates (Costar, Cambridge, MA). Cells were incubated with 1 ml of media at 37⁰ C in a tissue culture incubator equilibrated with 95% air and 5% CO₂. At near confluence, the cells were treated with EGCG at various concentrations (0 µg/ml, 10 µg/ml, 20 µg/ml, and 50 µg/ml), with and in the absence of LPA (a combination of 400 µM lysine, 140 µM proline and 100 µM ascorbic acid (Sigma); each dose was tested in triplicate. The plates were then returned to the incubator. The cells were washed with phosphate buffered saline (PBS) and 500 µl of MTT (Sigma #M-2128) 0.5 mg/ml in media was added to each well. Cytotoxicity was evaluated 24 hrs following incubation with test reagents. Culture media components were

purchased from Gibco (Grand Island, NY). All other chemicals used were purchased from Sigma (St. Louis) and were of high quality.

MTT assay

Viability/cytotoxicity was evaluated based on cell proliferation by MTT assay. The MTT assay [Masman (1983)] is a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide] (MTT) to a blue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells. This test is a good index of mitochondrial activity and thus of cell viability. After MTT addition (0.5mg/ml) the plates were covered and returned to the 37°C incubator for 2 hours, the optimal time for formazan product formation. Following incubation, the supernatant was carefully removed from the wells, the formazan product was dissolved in 1 ml DMSO, and absorbance was measured at 570 nm in Bio Spec 1601, Shimadzu spectrometer. The OD₅₇₀ of the DMSO solution in each well was considered to be proportional to the number of cells. The OD₅₇₀ of the control (treatment without supplement) was considered 100%.

Gelatinase zymography

MMP expression in condition media was determined by gelatinase zymography. Gelatinase zymography was performed in 10% Novex precast SDS-polyacrylamide gel (Invitrogen Corporation) in the presence of 0.1% gelatin under non-reduced conditions [Ballin, Gomez *et al.*(1988)]. Culture media (20 µl) mixed with sample buffer was loaded and SDS-PAGE was performed with tris glycine SDS buffer as described by the manufacturer (Novex). Samples were not boiled before electrophoresis. Following electrophoresis the gels were washed twice in 2.5% Triton X-100 for 30 minutes at room temperature to remove SDS. The gels were then incubated at 37°C overnight in substrate buffer containing 50 mM Tris-HCl and 10 mM CaCl₂ at pH 8.0 and stained with 0.5% Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid for 30 minutes and destained. Protein standards were run concurrently and approximate molecular weights were determined by plotting the relative mobilities of known proteins.

Matrigel invasion studies

Invasion studies were conducted using Matrigel™ (Becton Dickinson, Franklin Lakes, NJ) matrix-coated 9-mm cell culture inserts (pore size, 8 µm;); set in 24-well plates

using a modified Boyden Chamber method as described by Albini [Albini, Iwamoto *et al* (1987)]. 200 μ l of cell suspension (3×10^4 cells) supplemented with nutrients, as specified in the design of the experiment in triplicate, were seeded on the insert in the well. The lower chambers also contained 5% fetal bovine serum as a chemoattractant. The plates with the inserts were then incubated in a culture incubator equilibrated with 95% air and 5% CO₂ for 24 hours

After incubation, the remaining cells in the upper layer of the insert were carefully swabbed with cotton. The penetrating cells in the lower layer were fixed with cold methanol and stained with hematoxylin and eosin. The cells that invaded the lower side of the filter were counted using optical microscope.

Statistical analysis

The results were expressed as means \pm SE for the groups. Data was analyzed by independent sample “t” test.

Results

Fibrosarcoma cytotoxicity/proliferation study

Addition of LPA (400 μ g/ml lysine, 140 μ g/ml proline and 100 μ g/ml ascorbic acid) to various concentrations of EGCG (10 μ g/ml, 20 μ g/ml and 50 μ g/ml) enhanced the cytotoxic effect seen at those concentrations of EGCG alone. Furthermore, both EGCG alone and the combination of LPA and EGCG exhibited dose-response toxicity with a maximum toxicity of 44.8 % over the control when treated with LPA and 50 μ g/ml of EGCG ($p < 0.0001$). (See Figure 1.)

Gelatinase zymography study

As shown in Figure 2A, zymography demonstrated expression of MMP-2 and MMP-9 by human fibrosarcoma HT-1080 cells. The addition of LPA to 10 μ g/ml or 20 μ g/ml EGCG resulted in greater inhibition of MMP expression than in the presence of the same respective doses of EGCG alone. Nutrient synergy inhibited the expression of both MMPs in a dose-dependent fashion with virtual total inhibition at EGCG 50 μ g/ml + LPA. PMA stimulated increased expression of MMP-9, which also was inhibited by nutrient synergy in a dose-dependent fashion with virtual total inhibition at LPA + EGCG 50 μ g/ml (Figure 2B).

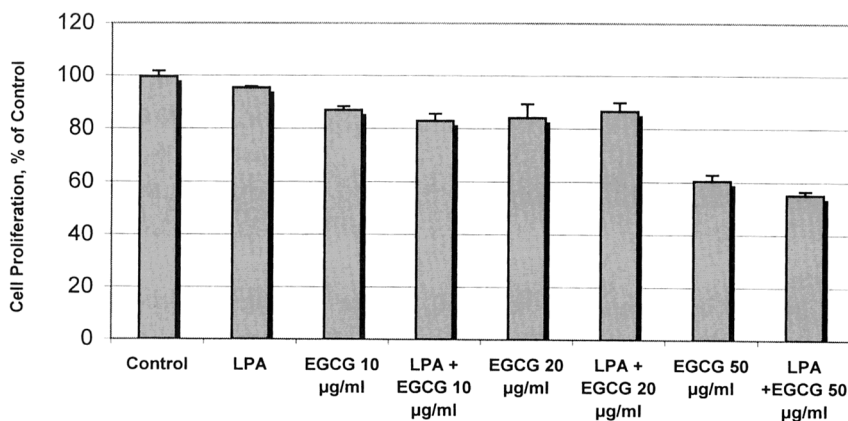


Figure 1 - Cytotoxicity study: effect of lysine (400 µg/ml), proline (140 µg/ml), ascorbic acid (100 µg/ml) and various levels of EGCG on fibrosarcoma HT-1080 proliferation ($p < 0.0001$)

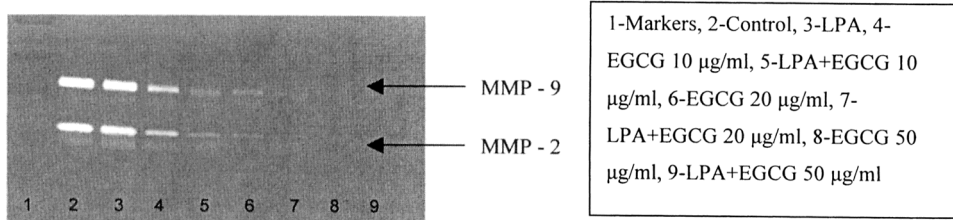


Figure 2A - Effect of lysine (400 µg/ml), proline (140 µg/ml), ascorbic acid (100 µg/ml) and EGCG on MMP-2 and MMP-9 expression by fibrosarcoma cells

Invasion study

The invasion of fibrosarcoma cells through Matrigel was reduced by 25% in the presence of LPA alone and 40% in the presence of EGCG 20µg/ml. However, the synergy of LPA + EGCG 20 µg/ml resulted in 63% inhibition of invasion. Total inhibition of invasion was achieved with cells in the presence of LPA + 50 µg/ml EGCG ($p=0.004$).

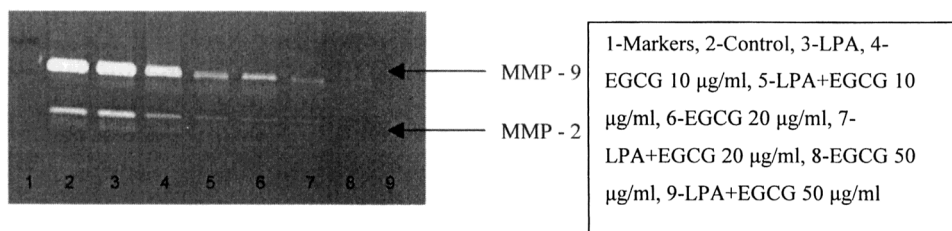


Figure 2B - Effect of lysine (400 µg/ml), proline (140 µg/ml), ascorbic acid (100 µg/ml) and EGCG on MMP-2 and MMP-9 expression by fibrosarcoma cells induced with PMA

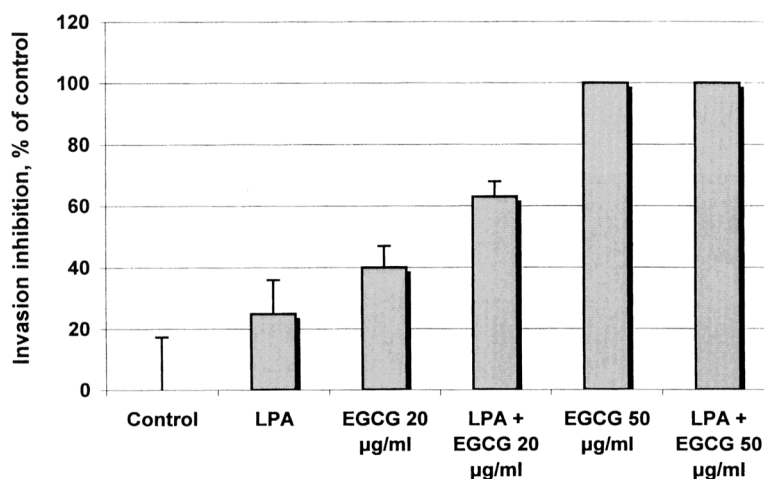


Figure 3 - Invasion Study: effect of lysine (400 µg/ml), proline (140 µg/ml), ascorbic acid (100 µg/ml) and various levels of EGCG on inhibition of Matrigel invasion and migration by fibrosarcoma HT-1080 cells (p = 0.004).

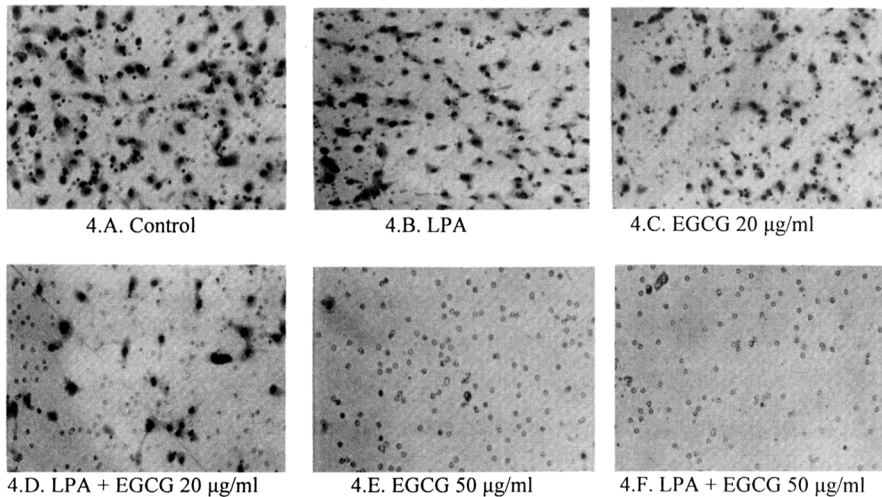


Figure 4- Invasion Study: Hematoxylin & Eosin Stained Photomicrographs (100x)

Discussion

The results of this study showed minimal toxicity to fibrosarcoma cells at dosages at and below EGCG 20 µg/ml +LPA without morphological changes (pictures not shown), and drastic anti-invasive effects of nutrient synergy *in vitro* on human fibrosarcoma cell line HT-1080. Matrigel invasion and expressions of MMP-2 and MMP-9 by fibrosarcoma cancer cells decreased in a dose-dependent fashion in the presence of EGCG, with enhanced inhibition of MMP expression and Matrigel invasion when LPA was added to the EGCG. Complete inhibition of invasion and MMP expression in both PMA stimulated and unstimulated cells was achieved in the presence of 50 µg/ml EGCG + LPA.

Certain MMPs, such as MMP-2 and MMP-9 are correlated with aggressiveness in human cancer and seem to play an important role in tumor invasion, angiogenesis and metastasis, especially in early stages of cancer [Zucker, Cao *et al.* (2000)]. Matrix invasion can be controlled by inhibition of MMP expression as well as by increasing connective tissue strength and stability, contributing to the “encapsulation” of the tumor. In this study, the increased inhibitory effect from the synergistic effect of LPA and EGCG on MMP-9 and MMP-2 expression by fibrosarcoma cells was consistent with its

inhibition of matrix invasion. In addition, matrix invasion was modulated by enhanced stability and strength of the connective tissue secondary to the activity of the mixture of nutrients provided.

Proper collagen formation is an important factor in the encapsulation of tumors or the slowing of metastasis via the development of an almost impermeable barrier. Lysine and proline are the building blocks of collagen fibers that stabilize connective tissues. Ascorbic acid is essential for hydroxylation of lysine and proline to form stable collagen, inhibition of hyaluronidase (maintaining ground substance around tumor intact), and is a scavenger of free radicals, protecting cells from damage. However, since it is not produced in the human body, sub-optimal levels are common and low levels of ascorbic acid have been reported in cancer patients [Anthony and Schorah (1982), Nunez, Ortiz de Apodace *et al* (1995), Kurbacher, Wagner *et al.* (1996)].

The inhibitory effects of the individual nutrients composing the test nutrient mixture have been reported in both clinical and experimental studies. In an *in vitro* study, human fibrosarcoma cells HT-1080 were treated with ASC-2-0-phosphate-6-O-palmitate (a lipophilic and auto-oxidation-resistant derivative of ascorbic acid), and in as little as 30 minutes, tumoral invasion was inhibited by 50%, with 80% inhibition achieved in 90 minutes, without cytotoxic effect. Additionally, zymography and Western blots showed significant inhibition of MMP-9 and MMP-2 expression, suggesting powerful chemopreventative and antimetastatic ability via potent antioxidant activity [Liu, Nagawa *et al.* (1999)]. EGCG, a green tea extract, provides antioxidant and anticarcinogenic activity. Other studies on the effect of tea polyphenols on MMP activities and invasion in human fibrosarcoma HT-1080 support our findings, showing that addition of EGCG to the cells significantly decreased invasion and suppressed gelatin degradation by MMP-2 and MMP-9 [Maeda-Yamamoto, Kawahara *et al.* (1999), Maeda-Yamamoto, Suzuki *et al.* (2003), Dell'Aica, Dona *et al.* (2002)].

However, individual nutrients are not as powerful as nutrient synergy. The results of this study showed EGCG had significant anti-proliferative and anti-invasive action on the fibrosarcoma cells HT-1080 tested, which were enhanced with addition of LPA to EGCG. In addition, our previous studies demonstrated that the synergistic anticancer effect of ascorbic acid, proline, lysine and EGCG on several cancer cell lines in tissue

culture studies was greater than that of the individual nutrients [Netke, Roomi *et al.* (2003)]. Furthermore, in contrast to chemotherapy which causes indiscriminate cellular and ECM damage, morphological studies (pictures not shown) demonstrated that even at the highest concentrations of nutrients tested, fibrosarcoma cells HT-1080, were not affected, demonstrating that this formulation is safe to cells.

The pharmaceutical industry has put great effort into developing MMP inhibitors for cancer treatment, such as: batimasta, marimastat, BAY12-9566, AG3340, COL-3, CT1746, KB-R7787 and several others and started many clinical trials. Despite two decades of research and billions of dollars spent, many of the clinical trials have failed because of adverse effects, such as anemia, anorexia, vomiting, fatigue and polyarthritis and lack of efficacy at lower more tolerable dosages [Sheehan, Bence *et al.* (2001)]. In contrast, we have demonstrated MMP inhibition and invasion using naturally occurring nutrients, such as lysine, proline, ascorbic acid and EGCG, which not only have no adverse effects, but support cellular health.

Conclusion:

Our results suggest that the nutrient mixture of EGCG, lysine, proline, and ascorbic acid is an excellent candidate for adjunctive therapeutic use in the treatment of the highly metastatic fibrosarcoma cancer, by inhibiting MMP expression and invasion while avoiding toxic effects.

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