

Original Article

In Vivo and In Vitro Antitumor Effect of Ascorbic Acid, Lysine, Proline, Arginine, and Green Tea Extract on Human Fibrosarcoma Cells HT-1080

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Abstract

Current treatment of fibrosarcoma, an aggressive cancer of the connective tissue, is generally associated with poor prognosis. Matrix metalloproteinases (MMPs), vascular endothelial growth factor (VEGF), and constituents of the extracellular matrix (ECM), such as fibronectin, play a critical role in angiogenesis and underlie neoplastic invasion and metastasis. This and anticancer properties of lysine, proline, arginine, ascorbic acid, and green tea extract (NM) prompted us to investigate the effect of these nutrients in vitro on human fibrosarcoma cells HT-1080 by measuring cell proliferation, modulation of MMP-2 and MMP-9, and invasive potential. In vivo, we studied the growth of human fibrosarcoma HT-1080 cells in athymic nude mice and the expression of MMPs and VEGF. Cell proliferation was evaluated by MTT assay, MMP expression by gelatinase zymography, and invasion through Matrigel and migration by scratch assay. Tumors were excised, weighed, and processed for histology in both the control and nutrient-supplemented groups. Results showed NM inhibited the growth and reduced the size of tumors in nude mice; decreased MMP-9 and VEGF secretion was found in the supplemented group tissues. NM inhibited invasion through Matrigel and migration with total inhibition at 1000 µg/mL. These results offer promise in the therapeutic use of the nutrient mixture of lysine, proline, arginine, ascorbic acid, and green tea extract tested in the treatment of fibrosarcoma.

Key Words: Fibrosarcoma; xenograft; nude mice; green tea extract; ascorbic acid; lysine; proline; antitumor effect; MMP-9; Matrigel invasion.

Introduction

Fibrosarcoma, an aggressive and highly metastatic cancer of the connective tissue, primarily develops in metaphyses of long tubular bones, and affects both children and adults (1). The poor prognosis associated with fibrosarcoma can be attributed to both the aggressive metastatic spread characteristic of this cancer and the lack of efficacy in current

treatment modalities to prevent, counteract, or slow tumor progression (2). Stage I fibrosarcoma is treated by surgical resection and may be treated postoperatively with radiotherapy or chemotherapy (3). However, even when fibrosarcoma is diagnosed and surgically treated early, current treatment is generally unsuccessful; the probability of the cancer metastasizing to distant parts of the body is greater than 70% after surgical treatment (1).

Chemotherapy and radiation therapy are the standard treatments for metastatic and recurrent fibrosarcoma; however, they have been ineffective in providing a cure (4). Both methods focus on cancer

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cell destruction, but do not address metastasis. Because these treatments are not cell specific, but indiscriminately attack all cells, cellular damage and destruction of the body's connective tissue result, facilitating metastasis. This helps to explain why individuals that have undergone radiation therapy carry a higher risk of developing fibrosarcoma (5). Furthermore, while knowledge of fibrosarcoma has improved over the last 20 years, survival has not changed, suggesting that current treatment methodologies have reached their limit of effectiveness (6). Taken together, these data suggest the need for new safe and effective therapeutic approaches that can be used to control the process of cancer metastasis.

Degradation of the basement membrane by matrix metalloproteinases (MMPs) is key to the invasive potential of cancer cells. The activity of these enzymes has been shown to correlate with the aggressiveness of tumor growth and invasiveness of the cancer. As postulated by Rath, nutrients such as lysine and ascorbic acid act as natural inhibitors of ECM proteolysis and, as such, have the potential to control tumor growth and expansion (7). These nutrients exercise their antitumor effect through the inhibition of MMPs and strengthening of connective tissue surrounding cancer cells. In a previous study, we demonstrated the antiproliferative and anti-invasive potential of lysine, ascorbic acid, proline, and green tea extract on human breast cancer (MDA-MB 231), colon cell cancer (HCT 116), and melanoma (A2058) cell lines (8). We investigated the effect of a nutrient mixture (NM) consisting of lysine, proline, arginine, ascorbic acid, and green tea extract on the growth of human fibrosarcoma xenografts in athymic nude mice. We also investigated the effect of NM on human fibrosarcoma cells HT-1080 in vitro, by measuring cell proliferation, modulation of MMP-2 and MMP-9, and invasive potential.

Materials and Methods

In Vivo Study

Cancer Cell Lines and Culture

Human fibrosarcoma cells HT 1080 obtained from ATCC (American Type Culture Collection, Rockville, MD) were maintained in MEM culture, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. The

media and sera used were obtained from ATCC, and antibiotics (penicillin and streptomycin) were from Gibco BRL (Long Island, NY). At near confluence, the cultured cells were detached by trypsinizing, washed with PBS, and diluted and emulsified to a concentration of 3×10^6 cells in 0.2 mL PBS and 0.1 mL Matrigel (BD Bioscience, Bedford, MA) for inoculation.

Animals

Male athymic nude mice (NCR-nu/nu), approx 6 wk of age on arrival, were purchased from Simonsen Laboratories (Gilroy, CA) and maintained in microinsulator cages under pathogen-free conditions on a 12-h light/12-h dark schedule for a week. All animals were cared for in accordance with institutional guidelines for the care and use of experimental animals. After housing for a week, the mice were inoculated with 3×10^6 human fibrosarcoma 1080 cells in 0.2 mL of PBS and 0.1 mL of Matrigel. After injection, the mice were randomly divided into two groups, A and B. Six mice were allocated to each group. From d 1, mice from Group A were fed a regular diet and those in Group B were fed a regular diet supplemented with 0.5% NM. After 4 wk, mice were sacrificed, tumors were excised, weighed, fixed in 10% (v/v) buffered formalin, and processed for histology.

Immunohistochemistry

Standard immunohistochemical staining procedures were used for staining antibodies. After deparaffinization and appropriate epitope retrieval, the sections were incubated with primary antibody. Detection was by biotinylated goat anti-mouse antibodies followed by streptavidin conjugated to horseradish peroxidase with the use of diaminobenzidine as the chromogen. Polyclonal rabbit anti-human antibodies used for MMP-9 and VEGF were obtained from Santa Cruz Biotechnology, Inc., and from Sigma.

In Vitro Study

Cell Culture

Human fibrosarcoma cells HT-1080 were obtained from ATCC (American Type Culture Collection) 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 37°C in a tissue culture incubator equilibrated with 95% air and 5% CO₂. At near confluence, the cells were treated with the nutrient mixture (NM) dissolved in media and tested in triplicate at 0 µg/mL, 10 µg/mL, 100 µg/mL, 200 µg/mL, and 1000 µg/mL. The plates were then returned to the incubator. The cells were washed with phosphate-buffered saline (PBS) and 500 µL of MTT (Sigma cat. no. M-2128), 0.5 mg/mL in media was added to each well. Proliferation was evaluated 24 h following incubation with test reagents. Culture media components were purchased from Gibco. All other chemicals used were purchased from Sigma.

MTT Assay

Viability/cell proliferation was evaluated by MTT assay (10), 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 (0.5 mg/mL), the plates were covered and returned to the 37°C incubator for 2 h, 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 a 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 (11). 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 min at room temperature to

remove the 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 min 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 et al. (12); 200 µL 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 h. 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 an optical microscope.

Scratch/Migration Assay

Human fibrosarcoma cells HT-1080 were cultured in 24-well tissue culture plates. At near confluence, a 2-mm-wide single uninterrupted scratch from top to bottom of culture plates was made. Culture plates were washed with PBS and incubated with nutrient mixture (NM) 0 µg/mL, 10 µg/mL, 50 µg/mL, 100 µg/mL, 500 µg/mL, and 1000 µg/mL in triplicate at each dose and incubated for 24 h. The cells were washed with PBS, fixed, and stained with H&E. Photomicrographs 100× were taken.

Nutrient Mixture (NM) Composition

The stock solution of the nutrient mixture (total weight 4.2 g) is composed of the following: vitamin C (as ascorbic acid and as Mg, Ca, and palmitate ascorbate) 700 mg; L-lysine 1000 mg; L-proline 750 mg; L-arginine 500 mg; N-acetyl cysteine 200 mg; standardized green tea extract 1000 mg (green tea

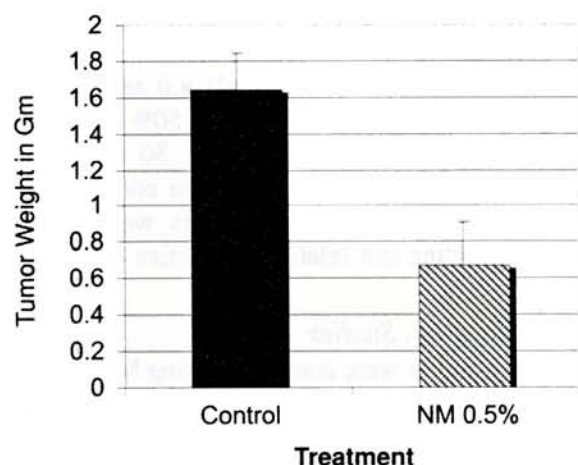


Fig. 1. Tumor growth study. Effect of combination of lysine, proline, arginine, ascorbic acid, and green tea extract (NM) on tumor size in nude mice ($p = 0.0001$).

extract derived from green tea leaves was obtained from US Pharma Lab; the certificate of analysis indicates the following characteristics: total polyphenol 80%, catechins 60%, EGCG 35%, and caffeine 1.0%; selenium 30 mg; copper 2 µg; manganese 1 mg.

Statistical Analysis

The results were expressed as means \pm SD for the groups. Data were analyzed by independent sample "t" test.

Results

In Vivo Study

Tumor Growth

Results showed that the nutrient-supplemented nude mice developed significantly smaller tumors (by 59%, $p = 0.0001$) and less vascular ones than did the control group of nude mice (Fig. 1). Animal weight did not decrease in either group during the study.

Immunohistochemistry

Control tissue showed slightly greater stained material for the following: VEGF (Figs. 2A, B) and MMP-9 (Figs. 2C, D).

In Vitro Study

Fibrosarcoma Proliferation Study

The nutrient mixture (NM) showed no inhibition of human fibrosarcoma cell HT-1080 proliferation at

100 µg/mL, slight antiproliferative effect at 200 µg/mL and 500 µg/mL, and a maximum of 37% at 1000 µg/mL ($p = 0.0005$). See Fig. 3.

Gelatinase Zymography Study

As shown in Fig. 4, human fibrosarcoma HT-1080 cells demonstrated expression of MMP-2 greater than MMP-9, both of which were inhibited by the nutrient mixture in a dose-dependent fashion, with virtual total inhibition of MMP-9 at 100 µg/mL and nearly total inhibition of MMP-2 at 1000 µg/mL.

Invasion Study

Invasion of fibrosarcoma cells through Matrigel was reduced by 40% in the presence of 10 µg/mL of the nutrient formulation (42%) and totally inhibited at 1000 µg/mL ($p = 0.0001$). See Figs. 5A, B.

Scratch Assay

NM reduced fibrosarcoma HT 1080 cell migration by scratch test after 24 h in a dose-dependent fashion with total inhibition at 500 µg/mL (Figs. 6A–H).

Discussion

The results of this study demonstrated significant inhibition of tumor growth of human fibrosarcoma xenografts in nude mice by supplementation with the 0.5% nutrient formulation. Histological analysis identified a slight decrease in MMP-9 and VEGF material in the supplemented group; the in vitro studies demonstrated significant anti-invasive effects of NM on human fibrosarcoma cell line HT-1080. Matrigel invasion and migration and expressions of MMP-2 and MMP-9 by fibrosarcoma cancer cells decreased in a dose-dependent fashion in the presence of NM, with complete inhibition of invasion and MMP-9 expression at 1000 µg/mL, of migration at 500 µg/mL, and of MMP-2 at 100 µg/mL.

A promising approach to cancer is targeting universal pathomechanisms involved in cancer growth and invasion. Cancer invasiveness can be blocked by tumor encapsulation, through a decrease in matrix degradation accompanied by optimized ECM structure and integrity. Degradation of basement membranes by MMPs is key to the invasive potential of cancer cells. Research has shown that highly metastatic cancer cells secrete higher amounts of MMPs than do poorly metastatic cells, demonstrating that the invasive and

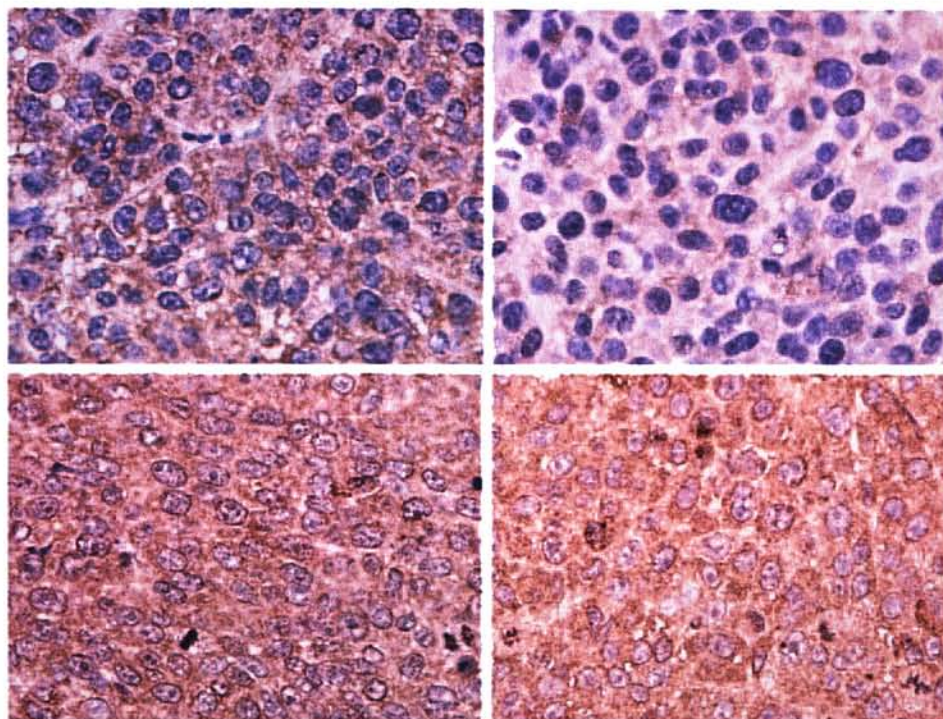


Fig. 2. Effect of nutrient supplementation (NM) on immunohistochemistry of tumor tissues from nude mice with fibrosarcoma HT-1080 xenografts ($\times 100$ magnification): (A) VEGF-control; (B) VEGF-NM 0.5%; (C) MMP-9-control; (D) MMP-9-NM 0.5%.

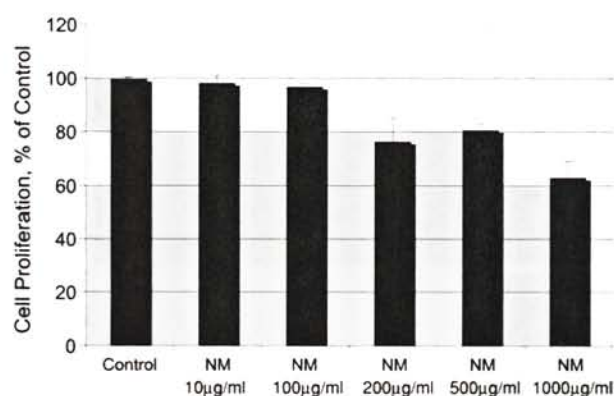


Fig. 3. Cell proliferation study: effect of combination of lysine, proline, arginine, ascorbic acid, and green tea extract (NM) on fibrosarcoma HT-1080 cells ($p < 0.0005$).

metastatic abilities of these cancer cells in vitro and in vivo correlate with MMP-9 expression (13). Control of proteolytic activity of ECM provides an opportunity of



Fig. 4. Effect of combination of lysine, proline, arginine, ascorbic acid, and epigallocatechin gallate (NM) on MMP-2 and MMP-9 expression by fibrosarcoma cells. Lanes correspond as follows: 1-markers, 2-control, 3-7 NM 10, 50, 100, 500, 1000 µg/mL.

addressing common mechanism of metastasis, angiogenesis, and tumor growth. Rath and Pauling (7), suggested targeting plasmin-mediated mechanisms with the use of nutritional components, such as lysine and lysine analogs. Lysine interferes with the activation of

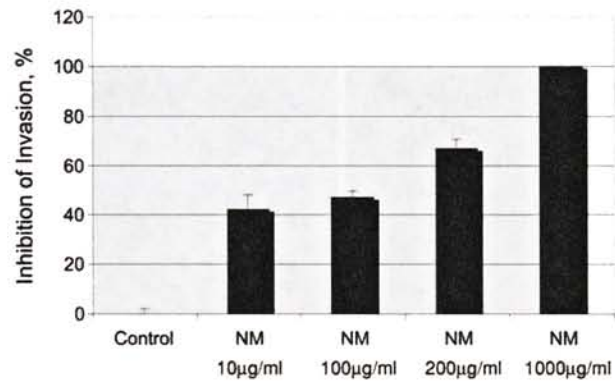


Fig. 5. Invasion study: effect of combination of lysine, proline, arginine, ascorbic acid, and green tea extract (NM) on inhibition of Matrigel invasion and migration by fibrosarcoma HT-1080 cells ($p = 0.0001$).

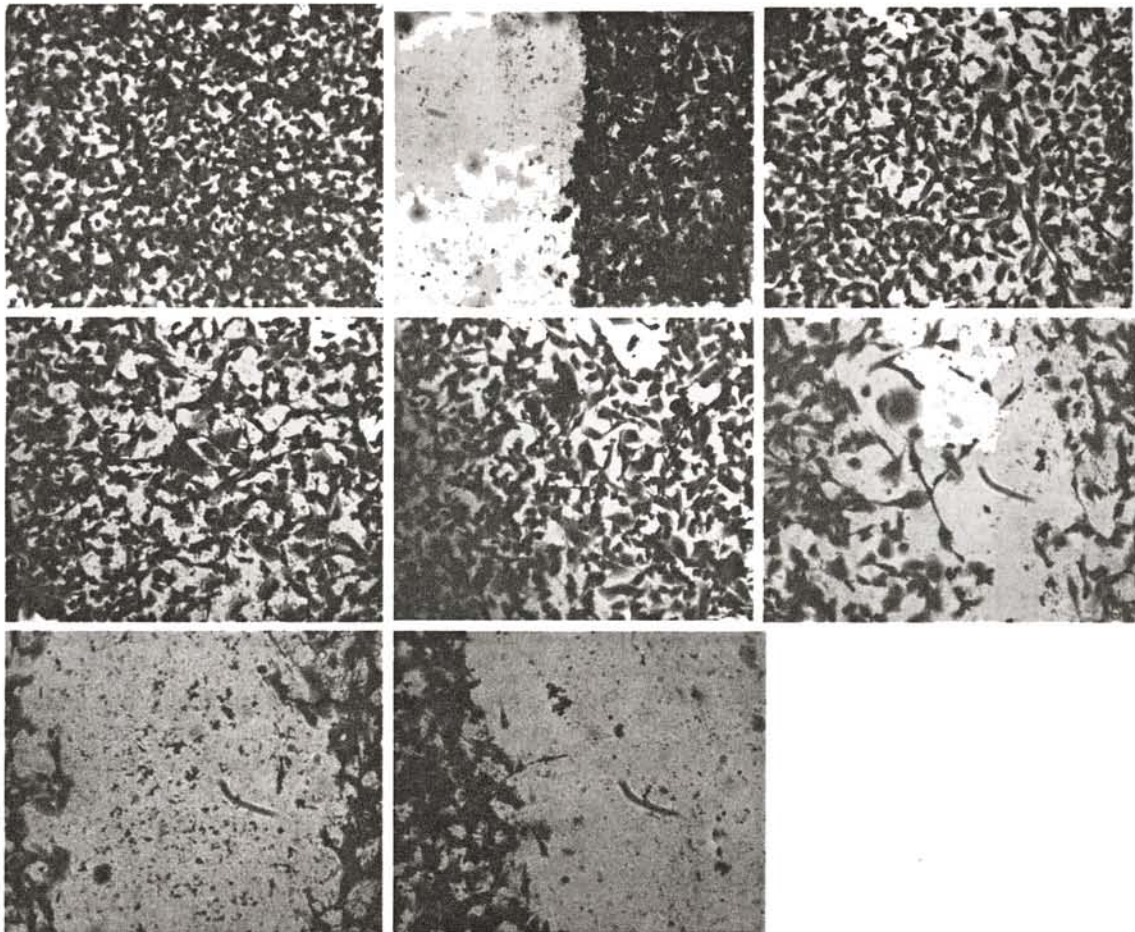


Fig. 6. Migration study: effect of combination of lysine, proline, arginine, ascorbic acid, and green tea extract (NM) on inhibition of scratch migration: (A) Prescratch; (B) Scratch; (C) Control; (D) NM 10 µg/mL; (E) NM 50 µg/mL; (F) NM 100 µg/mL; (G) NM 500 µg/mL; (H) NM 1000 µg/mL.

plasminogen into plasmin by tissue plasminogen activator (tPA) by binding to plasminogen active sites, and consequently affecting the plasmin-induced MMP activation cascade (7). A recent study demonstrated sevenfold reduction in metastasis of transgenic mammary cancer in plasmin-deficient mice (14). Lysine-mediated effects on the ECM include increased connective tissue strength and stability. It is well known that optimization of synthesis and structure of collagen fibrils depends on hydroxylation of proline and lysine residues in collagen fibers catalyzed by ascorbic acid. Suboptimal levels of ascorbic acid and lysine are possible in various pathological stages and in deficient diets, as these nutrients are not produced in the human body.

The inhibitory effects of the individual nutrients composing the nutrient mixture have been reported in both clinical and experimental studies. Low levels of ascorbic acid have been reported in cancer patients (15–17). In an *in vitro* study, human fibrosarcoma cells HT-1080 were treated with ASC-2-O-phosphate-6-O-palmitate (a lipophilic and autooxidation-resistant derivative of ascorbic acid), and in as little as 30 min, tumoral invasion was inhibited by 50%, with 80% inhibition achieved in 90 min, 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 (18). Other studies on the effect of tea polyphenols on MMP activity and invasion in human fibrosarcoma HT-1080, showing that addition of EGCG to the cells significantly decreased invasion and suppressed gelatin degradation by MMP-2 and MMP-9 (19–21), support our findings.

However, individual nutrients are not as powerful as nutrient synergy. The results of a previous study on fibrosarcoma showed enhanced anti-invasive action on the fibrosarcoma cells HT-1080 tested in the presence of EGCG, lysine, proline, and ascorbic acid over that seen with EGCG alone at the same concentration. In addition, other 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 (9). Furthermore, in contrast to chemotherapy, which causes indis-

criminate cellular and ECM damage, morphological studies showed that even at the highest concentrations of nutrients tested, fibrosarcoma cells HT-1080, were not affected, demonstrating that this formulation is safe to cells.

While clinical studies are necessary to better determine the efficacy of nutrient therapy in both cancer prevention and treatment, the results of this study suggest the formulation of lysine, proline, arginine, ascorbic acid, and epigallocatechin gallate tested as an excellent candidate for adjunctive therapeutic use in the treatment of the highly metastatic fibrosarcoma cancer, by inhibiting MMP expression and invasion without toxic effects.

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