

in vivo

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***In Vivo and In Vitro* Antitumor Effect of Ascorbic Acid, Lysine, Proline and Green Tea Extract on Human Melanoma Cell Line A2058**

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Abstract. *Background: Melanoma, a very serious form of skin cancer, causes the most skin cancer-related deaths, due to metastasis. Structural changes in the extracellular matrix (ECM) are necessary for cell migration during tissue remodeling. MMPs, VEGF, Ki-67 (proliferative protein) and constituents of ECM play a critical role in angiogenesis, and are crucial in neoplastic invasion and metastasis. Materials and Methods: The effect of a diet (NM) containing lysine, proline, arginine, ascorbic acid and green tea extract on the growth of tumors induced by implanting human melanoma A2058 cells in athymic nude mice was examined and, also, on the expression of MMPs, VEGF and Ki-67 in these tumors. The effect of NM in vitro on the melanoma A2058 cell line was tested by measuring: cell proliferation by the MTT assay, expression of MMPs by gelatinase zymography and invasion through Matrigel. Results: Nutrient supplementation strongly suppressed the growth of tumors (by 57%) without adverse effects in nude mice. Histological studies supported these findings by showing inhibition of MMP-9 and VEGF secretion and mitotic index. In vitro, NM inhibited melanoma cell growth by 64% at 500 µg/ml and Matrigel invasion by 95% at 100 µg/ml NM. Conclusion: These results suggest that NM may have a therapeutic potential in melanoma.*

Melanoma, a very serious form of skin cancer, accounts for only 4% of all skin cancer cases; however, it causes the most skin cancer-related deaths. The number of new melanomas diagnosed in the United States is increasing; since 1973, the incidence rate for melanoma has more than doubled. The American Cancer Society estimates that around 55,000 new

melanomas will be diagnosed in the United States during 2004 (1). Though often curable in its early stages, melanoma may metastasize to other areas of the body, such as the lymph nodes, lungs, liver, brain or bones.

The activity of matrix metalloproteinases (MMPs) on the degradation of the extracellular matrix (ECM) plays a critical role in the formation of tumors and metastasis and has been found to correlate with the aggressiveness of tumor growth and invasiveness of the cancer (2). Earlier work by Rath *et al.* (3) defined common patho-mechanisms for all cancers, the destruction of ECM as a precondition for cancer cell invasion, growth and metastasis and suggested intervention through natural inhibitors of plasmin-induced proteolysis, such as lysine and its analogs. Our previous studies have confirmed the concepts described by Rath and Pauling (3) and resulted in identifying a novel formulation containing lysine, ascorbic acid, arginine, proline and green tea extract (NM) which has shown significant anticancer activity against a large number of cancer cell lines, blocking cancer growth, tissue invasion and MMP expression both *in vitro* and *in vivo*. We demonstrated the antiproliferative and anti-invasive potential of NM *in vitro* on human osteosarcoma cell lines U2OS and MNNG-HOS (4), breast cancer cell lines MDA MB 231 and MCF-7 (5), the colon cancer cell line HCT 116 (6) and the pancreatic cancer cell line PaCa-2 (7). Antitumoral potential was observed *in vivo* on human colon cancer cell HCT 116 xenografts in nude mice (8), human prostate cancer PC-3 xenografts in nude mice (9) and *N*-methyl-*N*-nitrosourea-induced mammary tumors in Sprague-Dawley rats (10).

In this study, the antitumor potential of a mixture containing ascorbic acid, lysine, proline, arginine and green tea extract (NM) was examined on human melanoma cells A2058 *in vivo* (xenograft in male nude mice) and *in vitro*.

Materials and Methods

Composition of the nutrient mixture (NM). The stock solution of the nutrient mixture used for testing was composed of the following:

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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 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%, epigallocatechin gallate (EGCG) 35% and caffeine 1.0%); selenium 30 mcg; copper 2 mg; manganese 1 mg.

In vivo studies

Cancer cell lines and culture: Human melanoma A2058 cells, obtained from ATCC (American Type Culture Collection, Rockville, MD, USA), were maintained in DMEM 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, USA). At near confluence, the cultured cells were detached by trypsinizing, washed with PBS and diluted and emulsified to a density of 3×10^6 cells in 0.2 ml PBS and 0.1 ml Matrigel (BD Bioscience, Bedford, MA, USA) for inoculation.

Animals: Male athymic nude mice (NCr-nu/nu), approximately six weeks of age on arrival, were purchased from Simonsen Laboratories (Gilroy, CA, USA) and maintained in microinsulator cages under pathogen-free conditions on a 12-hour light/12-hour dark schedule for a week. All the 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 melanoma 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 day one, 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 four weeks, the mice were sacrificed, the tumors were excised, weighed, fixed in 10% (v/v) buffered-formalin and processed for histology. The dimensions (length (L) x width (W)) of the tumors were measured with a pair of digital calipers, and the tumor value was calculated using the following formula: $\frac{1}{2} \times L \times W$. **Cytochemistry and immunohistochemistry:** Tissue samples were fixed in 10% buffered-formalin. All tissues were embedded in paraffin and cut at 4-5 microns. The sections were deparaffinized through xylene and graduated alcohol series to water and incubated for 5 minutes in aqueous 3% hydrogen peroxide to block endogenous peroxidase. Histological sections were stained with hematoxylin and eosin (H & E) and periodic acid-Schiff (PAS) stains for evaluation using a standard light microscope.

Immunochemical studies were performed on formalin-fixed, paraffin-embedded sections. 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, VEGF and Ki-67 were obtained from Santa Cruz Biotechnology, Inc., (CA, USA) and from Sigma.

In vitro studies

Cell culture: Human melanoma cells A2058 were obtained from ATCC and grown in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml) in 24-well

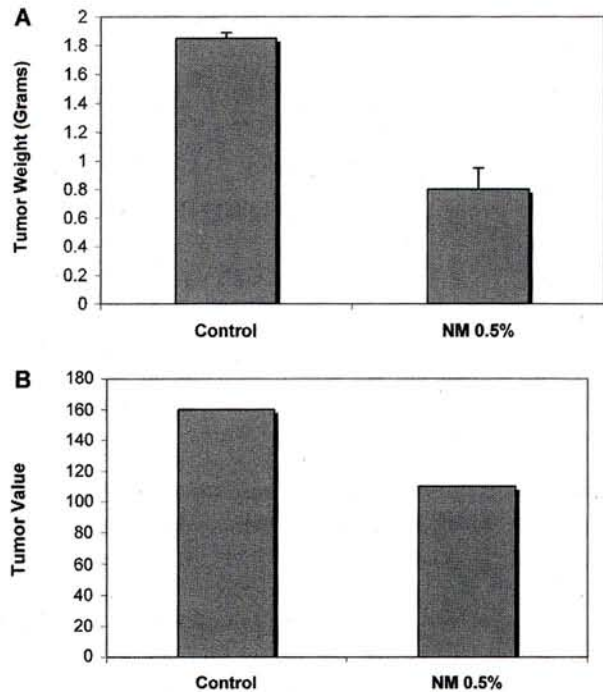


Figure 1. A. Effect of supplementation with 0.5% NM on mean tumor weight of melanoma xenografts in nude mice. B. Effect of supplementation with 0.5% NM on mean tumor value (in mm²) of melanoma A2058 xenografts in nude mice.

tissue culture plates (Costar, Cambridge, MA, USA). The 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 NM dissolved in media and tested at 10, 50, 100, 500 and 1000 mg/ml in triplicate at each dose. The plates were then returned to the incubator. Cell proliferation was evaluated after 24 hours following incubation with the test reagents.

MTT assay: The nutrient effects on cell proliferation were evaluated by MTT assay, 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. After MTT addition (0.5 mg/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 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 to be 100%.

Gelatinase zymography: MMP expression was determined by gelatinase zymography, which was performed in 10% polyacrylamide precast Novex gel (Invitrogen Corporation) in the presence of 0.1% gelatin. The culture media (20 µl) was loaded and SDS-PAGE was performed with a tris-glycine SDS buffer. After electrophoresis, the gels were washed with 5% Triton X-100 for 30 minutes. The gels were then incubated for 24 hours at 37°C in the

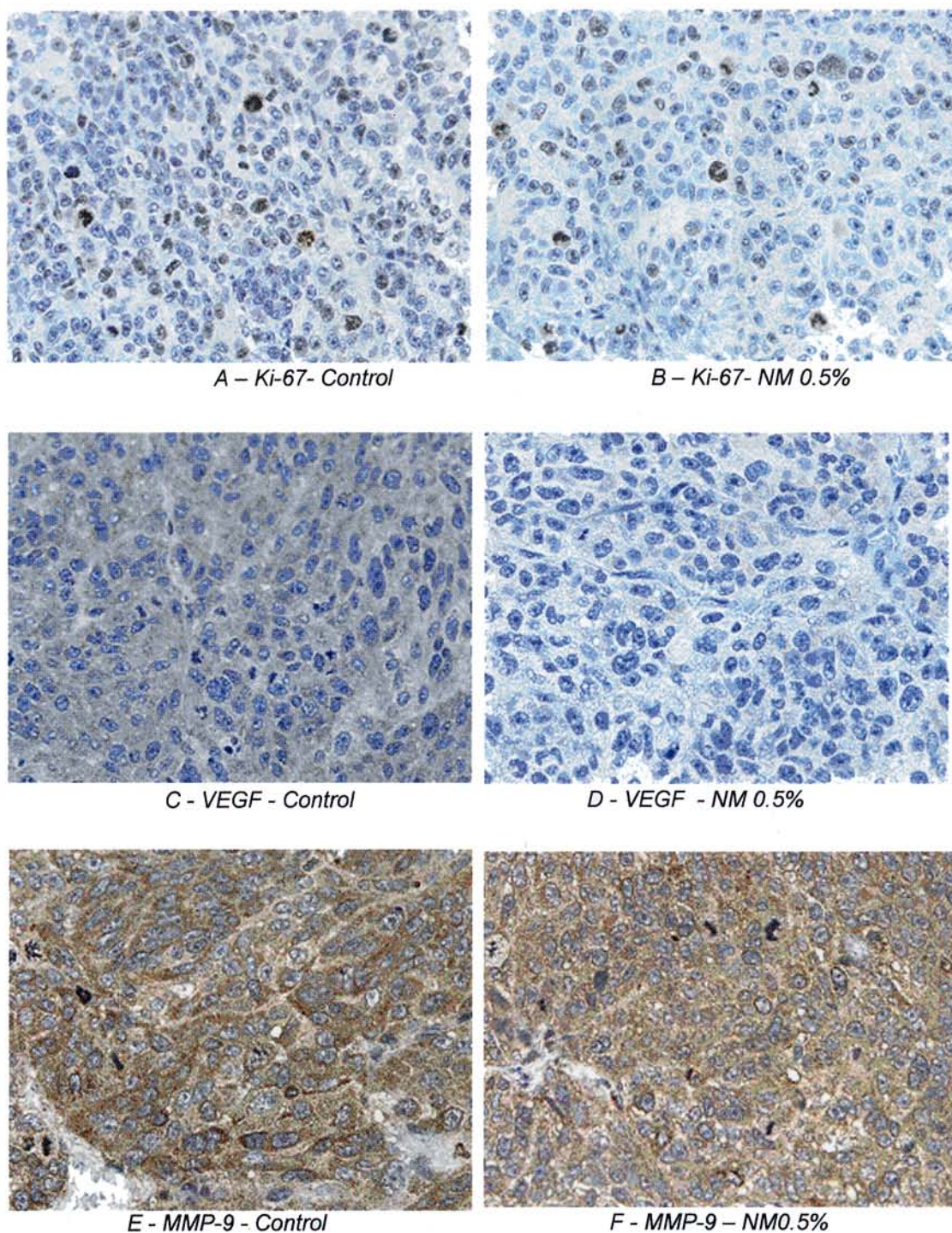


Figure 2. Effect of nutrient supplementation on melanoma A2058 xenograft tissue: cytochemistry and immunohistochemistry of control and supplemented tumor tissue: 2A – Ki-67 Control , 2B – Ki-67 NM 0.5%, 2C – VEGF Control, 2D – VEGF NM 0.5%, 2E – MMP-9 Control, 2F– MMP-9 NM 0.5%.

presence of 50 mM Tris-HCl, 5 mM CaCl₂, 5 μM ZnCl₂, pH 7.5 and stained with Coomassie Blue R 0.5% for 30 minutes and destained. Protein standards were run concurrently and approximate molecular weights were determined.

Matrigel invasion studies: Invasion studies were conducted using Matrigel™ (Becton Dickinson, Franklin Lakes, NJ, USA) matrix-coated 9-mm cell culture inserts (pore size 8 μm) set in 24-well plates, using a modified Boyden Chamber method as

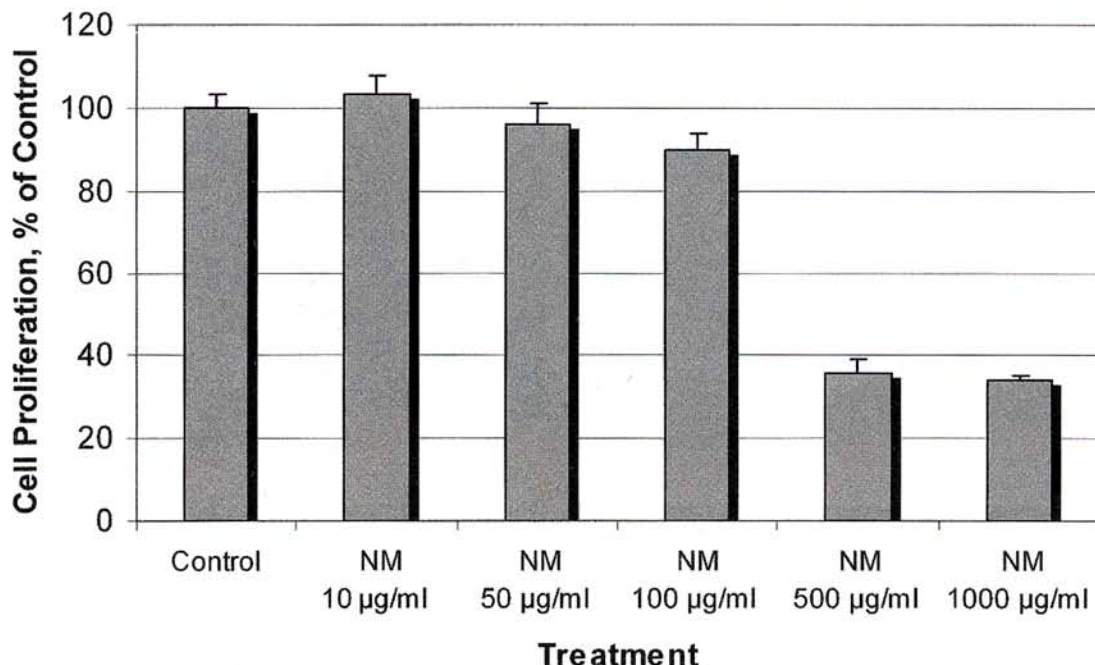


Figure 3. Effect of NM on melanoma A2058 cell proliferation: MTT assay.

described by Albini *et al.* (11). Two-hundred 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 hours. After incubation, the media from the wells were withdrawn. The cells on the upper surface of the inserts were gently scrubbed away with cotton swabs. The cells that had penetrated the Matrigel membrane and migrated onto the lower surface of the Matrigel were fixed with cold methanol, stained with H&E and visually counted using an optical microscope.

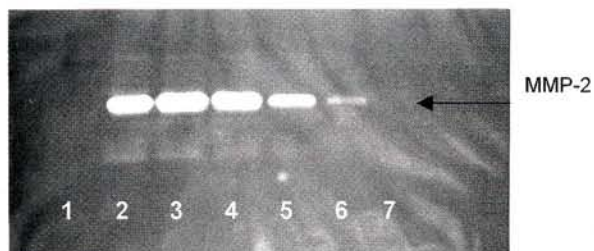
Statistical analysis. The results were expressed as means ± SD for the groups. The data was analyzed by independent sample *t*-test.

Results

In vivo studies

Tumor growth: The results showed that the nutrient-supplemented nude mice developed significantly smaller tumors (reduction in weight by 57%; $p < 0.0001$) and less vascular ones than did the control group of nude mice (Figure 1A). Nude mice from both groups showed no weight loss over the study period. Treatment with the nutrient formulation resulted in a significantly decreased mean tumor value (31%) in nude mice with human melanoma cancer cell xenografts studied over the four-week treatment period (Figure 1B).

A – Untreated cells



1-Marker, 2-Control, 3-7 NM 10, 50, 100, 500, 1000 µg/ml

B – PMA (200 ng/ml)-treated cells

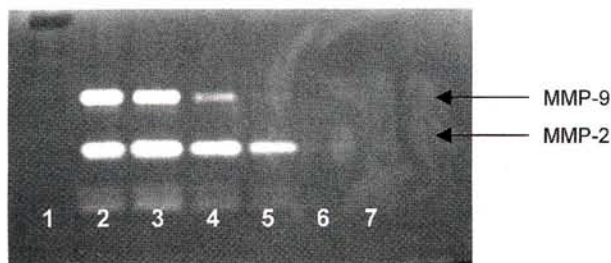


Figure 4. Effect of NM on melanoma A2058 MMP secretion: gelatinase zymography.

Cytochemistry and immunohistochemistry: The Ki-67 level, a measure of cell proliferation, was greater for the control group (Figures 2A-B). There is a strong positive correlation between

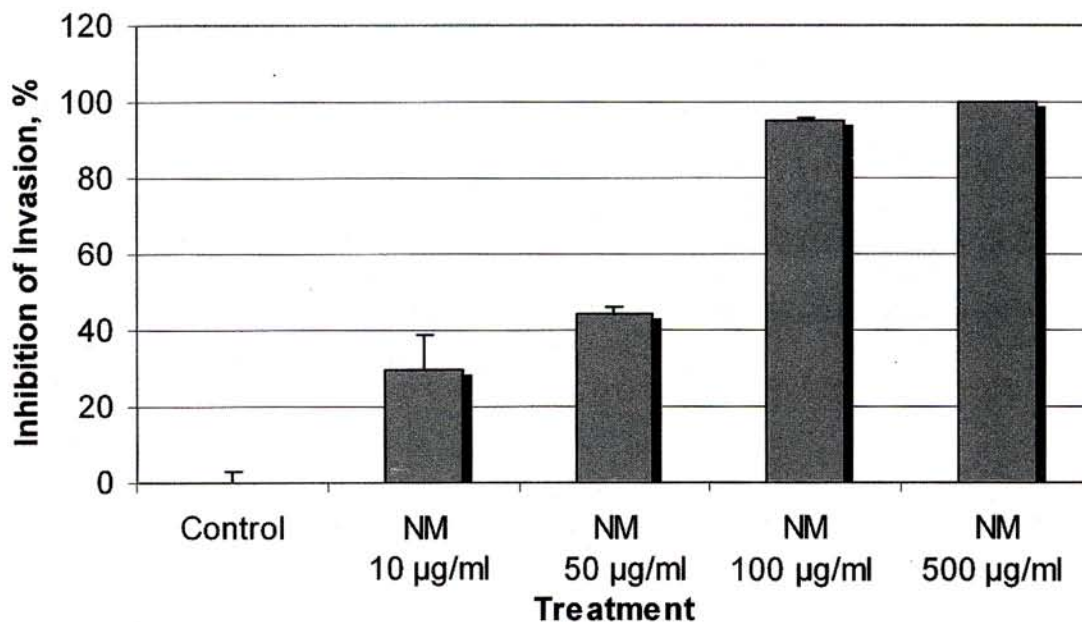


Figure 5. Effect of NM on Matrigel invasion of melanoma A2058 cells.

a high Ki-67 index and high-grade histopathology of neoplasms. VEGF staining, an indicator of angiogenesis, was higher in the control than in the supplemented group (Figures 2C-D). The control tissue cytoplasmic staining for MMP-9 was greater than in the supplemented mouse tissue (Figures 2E-F).

In vitro studies

Cell proliferation study: NM did not show significant antiproliferative effects on A2058 cells at 100 µg/ml but, at 500 µg/ml, inhibited cell growth by 64% ($p < 0.0001$, Figure 3).

Gelatinase zymography study: Zymography detected MMP-2 in the untreated melanoma A2058 cells (Figure 4A). Treatment of melanoma cells with phorbol 13-myristate, 12-acetate (PMA) (200 ng/ml) induced MMP-9 expression (Figure 4B). The nutrient mixture inhibited both MMP-2 and MMP-9 expression in a dose-dependent fashion with virtually complete blockage of MMP-9 at 100 µg/ml and MMP-2 at 500 µg/ml.

Matrigel invasion study: The invasion of melanoma cells through Matrigel was inhibited by 30%, 44%, 95% and 100% by 10, 50, 100 and 500 µg/ml of NM, respectively ($p < 0.0001$; Figures 5, 6).

Morphology (H&E): NM had no effect on the morphology of the melanoma cells even at the highest concentration.

Discussion

The results of this study demonstrated significant suppression of melanoma tumor growth in immune-impaired (athymic) male nude mice by supplementation with 0.5% of

the nutrient mixture (which contains ascorbic acid, lysine, proline, arginine and green tea extract). Histological examination demonstrated reduction in mitotic index and MMP-9 and VEGF material, as well as decreased PAS (mucin) material in the tissue of the supplemented animals. These results suggest that nutrient inhibition of tumor growth was associated with decreased cell proliferation and decreased angiogenesis and were supported by the *in vitro* results for these parameters. The nutrient mixture demonstrated a substantial antiproliferative effect on the human melanoma cancer cell line A2058 *in vitro* at 500 µg/ml and total suppression of MMP expression, Matrigel invasion and migration at 500 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 melanoma and other cancer cells secrete higher amounts of MMPs than do poorly metastatic cells, demonstrating that the invasive and metastatic abilities of these cancer cells *in vitro* and *in vivo* correlates with MMP-9 expression (12). Control of the proteolytic activity of ECM provides an opportunity of addressing common mechanisms of metastasis, angiogenesis and tumor growth. Rath and Pauling (3) suggested targeting plasmin-mediated mechanisms with the use of nutritional components, such as lysine and lysine analogs. Lysine interferes with the

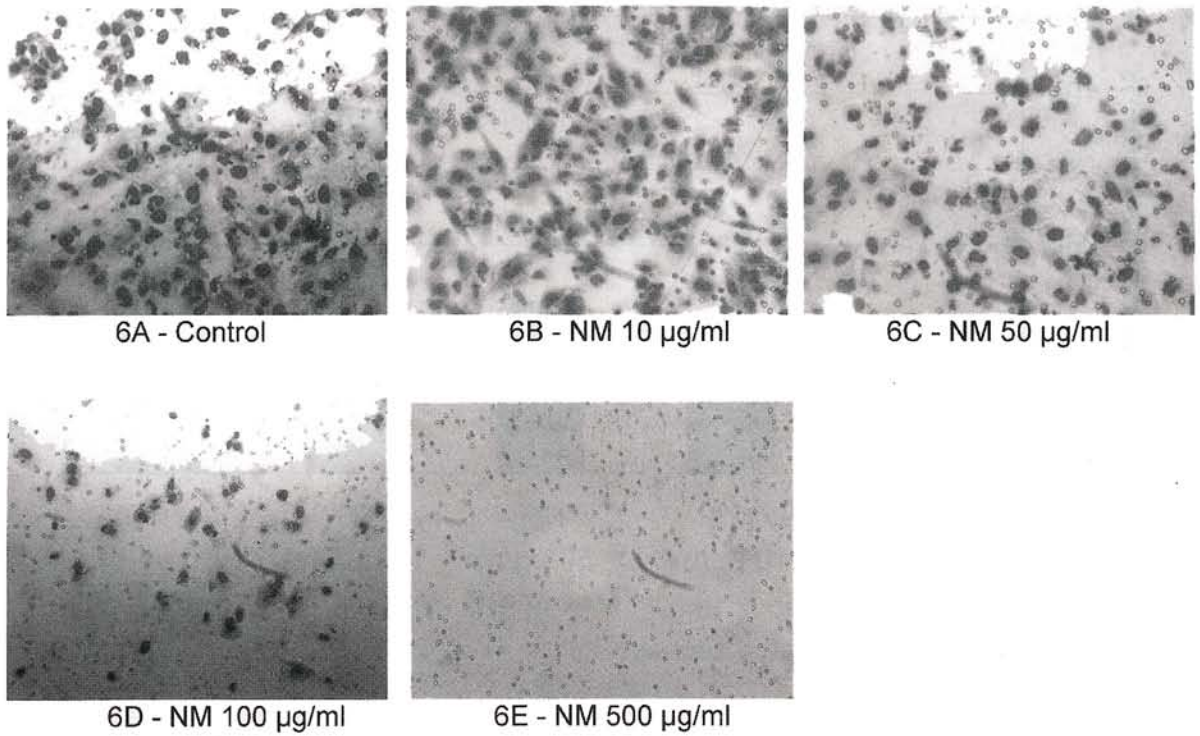


Figure 6. Invasion photomicrographs: 6A - Control, 6B - NM 50 µg/ml, 6C - NM 100 µg/ml, 6D - NM 500 µg/ml, 6E - NM 1000 µg/ml.

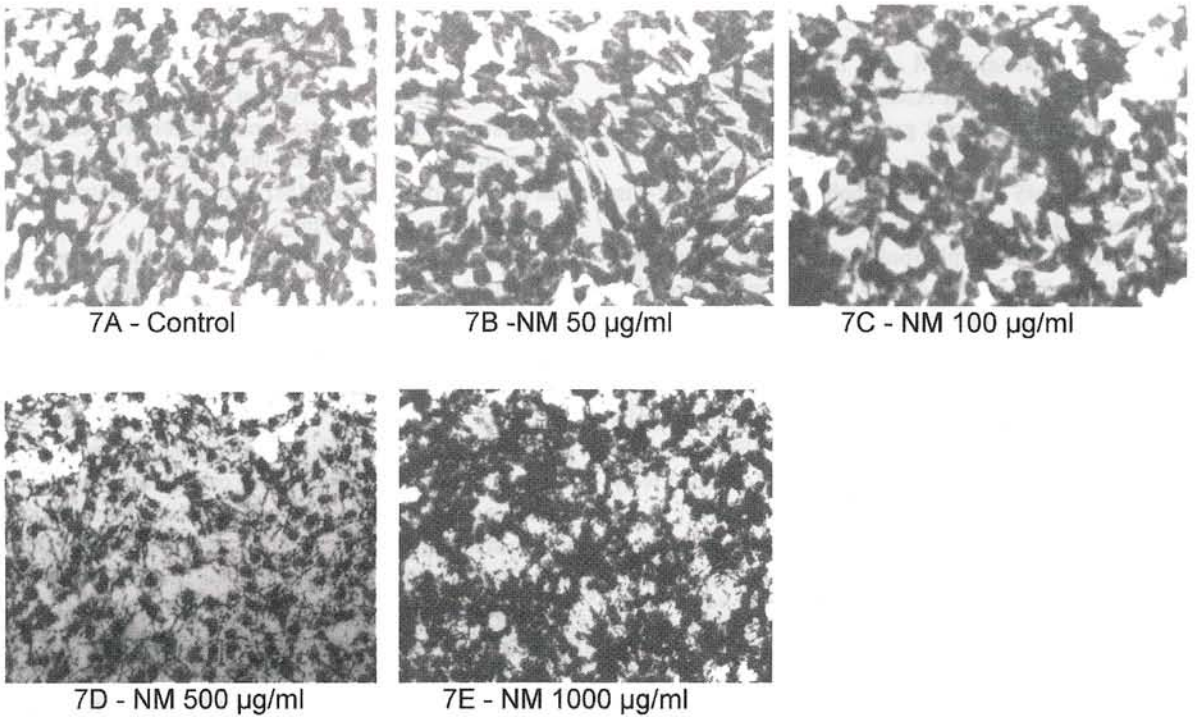


Figure 7. Effect of NM on melanoma A2058 morphology (H&E): 7A - Control, 7B - NM 50 µg/ml, 7C - NM 100 µg/ml, 7D - NM 500 µg/ml, 7E - NM 1000 µg/ml.

activation of plasminogen into plasmin by tissue plasminogen activator (tPA) by binding to plasminogen active sites, and consequently affecting the plasmin-induced MMP activation cascade (3). A recent study demonstrated a seven-fold reduction in metastasis of transgenic mammary cancer in plasmin-deficient mice (13). Lysine-mediated effects on the ECM include increased connective tissue strength and stability. It is well known that optimization of the synthesis and structure of collagen fibrils depends upon hydroxylation of proline and lysine residues in collagen fibers catalyzed by ascorbic acid. Sub-optimal levels of ascorbic acid and lysine are possible in various pathological stages and in deficient diets, since 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. Ascorbic acid has been reported to have cytotoxic and antimetastatic actions on malignant cell lines (14-16); in addition, low levels of ascorbic acid have been reported in cancer patients (17-19). Green tea extract is a potent anticancer agent that has been reported to have a growth inhibitory effect against melanoma and other human cancer cell lines (20-23). However, individual nutrients are not as powerful as nutrient synergy. 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 (24).

While clinical studies are necessary to better determine the efficacy of nutrient therapy in both melanoma prevention and treatment, the results of these studies suggest that the formulation of green tea extract, lysine, proline, arginine and ascorbic acid is an excellent candidate for adjunctive therapeutic use in the treatment of metastatic melanoma, by inhibiting MMP expression and invasion without toxic effects.

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