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IN VIVO AND IN VITRO ANTITUMOR EFFECT OF A UNIQUE NUTRIENT MIXTURE ON LUNG CANCER CELL LINE A-549

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□ *The high incidence of lung cancer and ineffective toxic action of current mono and doublet chemotherapy approaches result in poor patient survival. Further, matrix metalloproteinases (MMPs) are implicated in neoplastic invasion and metastasis. Based on this, the authors investigated the effect of a dietary micronutrient mixture (NM) containing lysine, proline, arginine, ascorbic acid, and green tea extract on the tumor growth of human lung carcinoma cell A-549 xenografts in athymic nude mice. Additionally, the authors tested the in vitro antitumor effect of NM on lung carcinoma A-549 cells by measuring cell proliferation by MTT assay, MMP-2 and -9 secretion by gelatinase zymography, and cell invasion through Matrigel. Nutrient supplementation strongly suppressed the growth of tumors without adverse effects in nude mice; tumor weight was reduced by 44% ($P = .0001$) and tumor burden was reduced by 47% ($P < .0001$) with supplementation. Zymography demonstrated in vitro secretion of MMP-2 by uninduced human lung carcinoma cells and both MMP-2 and -9 by phorbol 12-mysristate 13-acetate (PMA) (200 ng/mL)-treated cells. NM inhibited the secretion of both MMPs in a dose-dependent fashion, with virtual total inhibition at 500 μg/mL concentration. The invasion of human lung carcinoma cells through Matrigel was significantly reduced at 100 μg/mL (64%) and totally inhibited at 500 μg/mL concentration of NM ($P = .01$). Suppression of lung tumor growth in nude mice and inhibition of MMP secretion and Matrigel invasion suggest NM may act as an anticancer agent and as such warrants further investigation.*

Keywords A-549, antitumor effect, lung cancer, MMP, nutrient mixture

Lung cancer is the leading cause of cancer-related death for both men and women worldwide. For 2005, the American Cancer Society estimates approximately 172,570 new cases of lung cancer in the United States [1]. Worldwide incidence of lung cancer has increased from one million in 1999 to 1.35 million new cases in 2002 [2]. Despite a shift in treatment

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strategy in the 1970s from radiotherapy to sequential chemoradiation to concurrent chemoradiation, only marginal improvement in survival has been seen; nearly 163,510 Americans will die from lung cancer this year and 5-year survival is limited to 5% to 10% [3]. Average age at diagnosis is 70 and the American Cancer Society estimates that nearly 6 out of 10 people with lung cancer will die within 1 year of diagnosis. Non-small-cell lung cancer (NSCLC), a heterogeneous aggregate of histologies, comprising squamous, adenocarcinoma, and large-cell carcinoma, is the dominant histology responsible for roughly 80% of all lung malignancies [4]. Approximately 50% of NSCLC cases present with incurable metastatic (stage IV) disease [5]. Not only does radiation therapy and adjunctive chemotherapy fail to improve survival in NSCLC patients, but these strategies are associated with dose-dependent toxicities due to nonselective damage to cancerous and normal cells. Cisplatin is associated with severe nephrotoxicity [6]; vincristine, with dose-limiting neurotoxicity, manifested mainly as peripheral neuropathy [7]; doxorubicin with cardiotoxicity, which can lead to acute and chronic heart failure [8, 9]; etoposide with bone marrow suppression and gastrointestinal toxicity [10]; and cyclophosphamide with significant pulmonary toxicity [11]. Taken together, late diagnosis of lung cancer, high mortality, and the ineffective and harmful effects of chemotherapy and radiotherapy necessitate the adoption of novel treatment approaches that target and arrest metastasis.

Proteolytic degradation of the extracellular matrix (ECM) is necessary for cancer cells to invade and metastasize. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases implicated in facilitating tumor invasion and metastasis through perforation of physical barriers to invasion by degradation of ECM macromolecules and modulation of cell adhesion and activation of ECM components to expose hidden biologic activities. Due to this, recent research efforts have focused on the role of MMP inhibitors in the modulation of tumor invasion, metastasis, and angiogenesis. Rath and Pauling proposed that natural inhibitors, such as lysine and ascorbic acid, have the potential to inhibit tumor growth and expansion through the modulation of ECM proteolysis and optimization of connective tissue integrity [12]. Our earlier work based on this concept led to the development of a nutrient composition (NM) that has shown to be effective in achieving a complete inhibition of ECM invasion of several cancer cell lines in vitro, strong antimetastatic effects in animal models [13, 14], and demonstrated significant antiangiogenic and proapoptotic effects [15, 16].

In this report we investigated the antitumor potential of this NM-containing ascorbic acid, lysine, proline, arginine, and green tea extract on human lung carcinoma cells A-549 in vivo and in vitro.

MATERIALS AND METHODS

Cancer Cell Line and Culture

Human lung cancer cells A-549 obtained from ATCC (American Type Culture Collection, Rockville, MD) were maintained in Ham F12K medium, 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.

Composition of Nutrient Mixture

Stock solution of the NM was composed of the following in the ratio indicated: 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*-acetylcysteine 200 mg; standardized green tea extract (80% polyphenol) 1000 mg; selenium 30 μg; copper 2 mg; manganese 1 mg.

The NM was formulated based on targeting different physiological processes involved in cancer progression and metastasis at the cellular level. For example, the ECM integrity is dependent upon adequate collagen formation and its stability. In this aspect ascorbic acid and the amino acids lysine and proline are necessary for the formation and optimum structure of collagen fibers. Manganese and copper are also essential cofactors in collagen formation process. Collagen stability can be controlled by lysine [12] and also by *N*-acetylcysteine through its inhibitory effect on MMP-9 activity [17] and invasive activities of tumor cells [18]. Also, selenium has been shown to interfere with MMP expression and tumor invasion [19], as well as migration of endothelial cells through ECM [18]. Ascorbic acid, in addition to its critical role in ECM structure, has been shown to inhibit cancer cell division and growth through production of hydrogen peroxide, especially when applied in high doses [20, 21]. Green tea extract has shown to be a promising agent in controlling angiogenesis, metastasis, and other aspects of cancer progression [22]. Because arginine is a precursor of nitric oxide (NO), any deficiency of arginine can limit the production of NO, which has been shown to predominantly act as an inducer of apoptosis, as in the case of breast cancer cells [23].

Based on our own research and published data, we postulated that metabolic effects of a combination of ascorbic acid, lysine, proline, green tea extract, arginine, *N*-acetylcysteine, selenium, copper, and manganese would result from their synergy. For example, we found that a combination of ascorbic acid, lysine and proline used with epigallocatechin gallate (EGCG) enhanced the anti-invasive activity of 20 μg/mL EGCG to that of

50 μg/mL [24]. Thus by including nutrients such as *N*-acetylcysteine, arginine, selenium, manganese, and copper in addition to ascorbic acid, proline, lysine, and EGCG, we could obtain significant reduction in cell invasion at a much lower concentration of EGCG.

In Vivo Studies

Animals

Male athymic nude mice (NCr-nu/nu), approximately 6 weeks of age on arrival, were purchased from Simonsen Laboratories, Gilroy, CA, and maintained in microinsulator cages under pathogen-free conditions on a 12-hour light/12-hour 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 subcutaneously with 3×10^6 human lung cancer cells in 0.2 mL of phosphate-buffered saline (PBS) and 0.1 mL of Matrigel (BD Bioscience, Bedford, MA). After injection, the mice were randomly divided into 2 groups, A and B. Six mice were allocated to each group. From day 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 (*w*/*w*). During the study, the mice consumed, on the average, 4 g of their respective diets per day. (Thus, the supplemented mice received approximately 20 mg of NM per day.) After 4 weeks, mice were sacrificed; tumors were excised, weighed, and measured. Dimensions [length (L) × width (W)] of the tumors were measured with a pair of digital calipers, and the tumor burden was calculated using the following formula: $1/2 \times L \times W$. Tumors were then fixed in 10% (*v*/*v*) buffered formalin and processed for histology.

Histology

Tissue samples were fixed in 10% buffered formalin. All tissues were embedded in paraffin and cut at 4 to 5 microns. 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) for evaluation using a standard light microscope.

In Vitro Studies

Cell Culture

At near confluence, the lung carcinoma A549 cells were treated with the NM, dissolved in medium and tested at 0, 10, 50, 100, 500, and 1000 μg/mL in triplicate at each dose. Cells were also treated with

200 ng/mL of phorbol 12-mysristate 13-acetate (PMA). Five microliters of a 40 μg/mL solution of PMA was added to 0.5 mL of medium. The plates were then returned to the incubator.

MTT Assay

Cell proliferation was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide] assay 24 hours following incubation with test reagents. The MTT assay is a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt (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. The cells were washed with PBS and 500 μL of MTT (Sigma catalog number M-2128), 0.5 μ/mL in medium, was added to each well. After MTT addition, 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 OD_{570} of the DMSO solution in each well was considered to be proportional to the number of cells. The OD_{570} of the control (treatment without supplement) was considered 100%.

Gelatinase Zymography

MMP secretion in conditioned medium was determined by gelatinase zymography. Gelatinase zymography was performed in 10% Novex precast sodium dodecyl sulfate (SDS)-polyacrylamide gel (Invitrogen Corporation) in the presence of 0.1% gelatin under nonreduced conditions. Culture medium (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 $CaCl_2$ at pH 8.0, 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) inserts in 24-well plates. Suspended in medium, human lung cancer A-549 cells were supplemented with nutrients, as specified in the design of the experiment and seeded on the insert in the well. Thus both the medium on the insert and in the well contained the same supplements.

The plates with the inserts were then incubated in a culture incubator equilibrated with 95% air and 5% CO_2 for 24 hours. After incubation, the medium 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 stained with Hematoxylin and Eosin and visually counted under the microscope.

Statistical Analysis

The results were expressed as means $\pm$ SD or SEM, as indicated in the results, for the groups. Data was analyzed by independent-sample t test.

RESULTS

In Vivo Studies

Tumor Growth

Nude mice supplemented for 4 weeks with NM in their diets, developed significantly smaller tumors than did the control nude mice. Tumors developed in NM supplemented mice demonstrated lower weight (by 44%, $P = .0001$, as shown in Figure 1*A*) and tumor burden (by 47%, $P < .0001$, as shown in Figure 1*B*) compared to those developed by the control group of mice. Nude mice from both groups showed no body weight loss over the study period. The initial mean weight of mice was 20.6 ± 0.7 g; the mean post treatment weight of the control group of mice was 23.2 ± 1.02 g and of the supplemented group was 21.3 ± 1.02 g.

Histology

Sections from irregularly round, focally invasive subcutaneous masses of the control group of mice were composed of nests and sheets of irregularly round to cuboidal epithelioid cells with irregularly round nuclei and variably vacuolated cytoplasm consistent with a carcinoma. Bluish staining material, probably mucin, was occasionally seen in the interstitial tissue of tumors. Mitotic figures averaged about 1 per high-power field. Foci of tumor necrosis infiltrated with neutrophils were variably disseminated in the tumor, involving about 10% of the masses. Tumor masses were surrounded by fibroblasts and a moderate mixed inflammatory cell infiltrate. The histology of masses from the supplemented group were similar to the control group, except the necrosis involved about 20% of the tumor masses and the inflammatory cell response surrounding the tumors consisted primarily of macrophages.

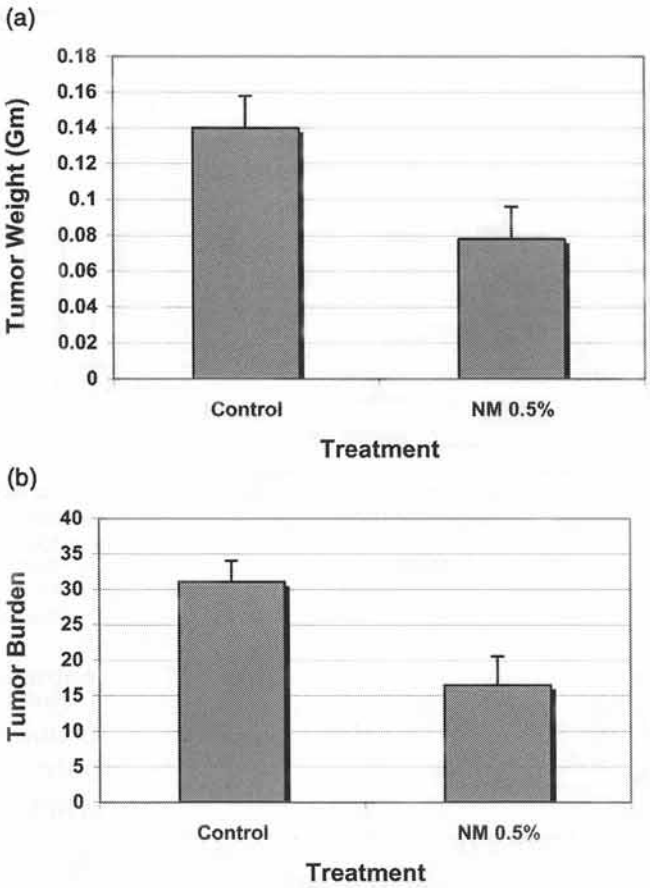

FIGURE 1 Effect of nutrient suplementation (NM 0.5% diet) on mean tumor weight (*A*) and mean tumor burden (*B*) in male nude mice (6 in each group) 4 weeks post inoculation with human lung cancer A-549 cell xenografts. Nutrient supplementation reduced tumor weight by 44% ($P = .0001$) and mean tumor burden by 47% ($P < .0001$). Error bars on graphs represent SEM. Tumor burden was calculated as $1/2 \times L \times W$.

In Vitro Studies

Cell Proliferation Study

The nutrient mixture applied at lower concentrations (up to 500 μg/ml) did not show any significant effect on lung carcinoma cell growth. Cell proliferation increased to 144% ($P = .002$) over the control at NM 500 μg/mL. However, at 1000 μg/mL, NM inhibited growth to 80% of the control ($P = .008$), as shown in Figure 2. It is difficult to explain the sudden decrease in cell proliferation from 500 to 1000 μg/mL. It is possible that the cells were not washed properly and that the residual NM interfered with the assay. However, current studies in progress have demonstrated

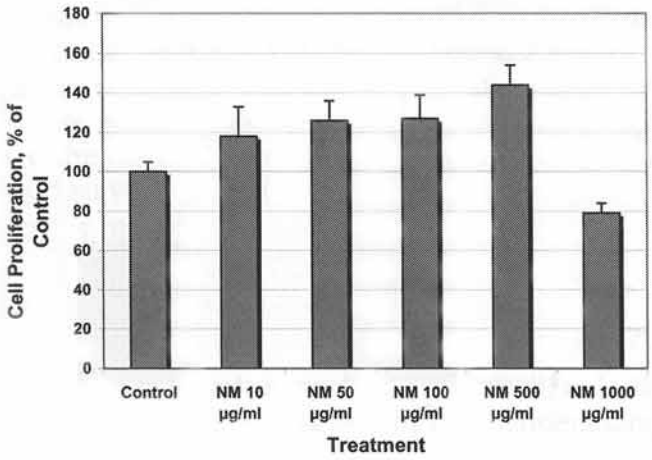

FIGURE 2 Effect of the nutrient mixture (NM) on cell proliferation of lung cancer A-549 cell line: MTT assay 24 h. NM showed minimal stimulation of A-549 cells up to 500 µg/mL, increased cell proliferation by 40% at 500 µg/mL, and reduction in cell growth to 80% of the control at 1000 µg/mL. Error bars represent SD.

increased apoptosis of A549 cells with higher NM concentration, especially at 500 and 1000 µg/mL.

Gelatinase Zymography Study

Zymography assays have shown that uninduced human lung carcinoma cells secrete only MMP-2 (Figure 3*A*), whereas PMA treatment (200 ng/mL) results in secretion of both MMP-2 and -9 (Figure 3*B*). Exposure of cells to NM resulted in inhibition of the secretion of both MMPs in a dose-dependent fashion, with virtual total inhibition at 500 µg/mL concentration.

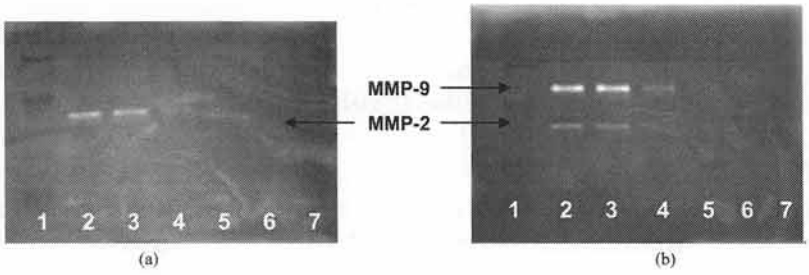

FIGURE 3 Effect of the nutrient mixture (NM) on lung carcinoma A-549 MMP-2 and MMP-9 secretion (*A*) and on PMA (200 ng/mL)-treated lung carcinoma A-549 MMP-2 and MMP-9 secretion (*B*). Lane 1, markers; lane 2, control; lanes 3–7, NM 10, 50, 100, 500, 1000 µg/mL. Zymography demonstrated secretion of MMP-2 and PMA-induced MMP-9 secretion. NM inhibited the secretion of both MMPs in a dose-dependent fashion with total inhibition at 500 µg/mL.

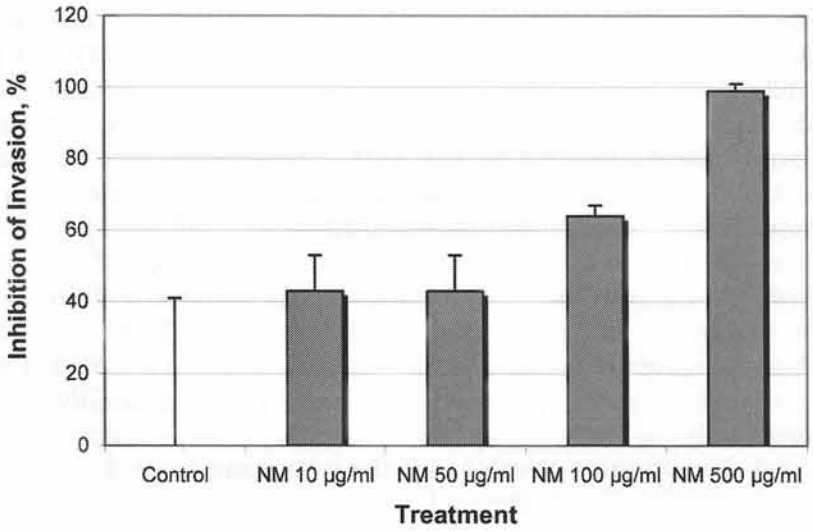

FIGURE 4 Effect of the nutrient mixture (NM) on lung cancer A-549 Matrigel invasion. NM inhibited Matrigel invasion of A-549 cells by 64% at 100 µg/mL and totally blocked it at 500 µg/mL NM ($P < .001$). Error bars represent SD.

Invasion Study

The invasion of human lung carcinoma cells through Matrigel was significantly reduced at 100 µg/mL (64%) and totally inhibited at 500 µg/mL concentration of the nutrient mixture ($P = .01$), as shown in Figure 4.

DISCUSSION

Matrix metalloproteinases are involved in tumor growth, angiogenesis, invasion, and metastasis. Tumor growth depends upon the generation of new blood vessels to sustain survival and proliferation. Growing tumors become hypoxic and acidotic beyond the size of 2 mm and secrete several growth factors to stimulate local blood vessels to sprout branches. Activation of endothelial cells give rise to the production of matrix metalloproteinases, allowing for the decomposition of the surrounding extracellular matrix and eventual development into new blood vessels.

The results of this in vivo study of human lung cancer cell xenografts in immune impaired (athymic) male nude mice demonstrated significant suppression of lung tumor growth (44% reduction in tumor weight and 47% reduction in tumor burden) with nutrient supplementation (NM 0.5%). In a previous study we showed that NM caused a significant ($P < .05$) reduction in basic fibroblast growth factor (bFGF)-induced angiogenesis in a chorioallantoic membrane (CAM) assay in chick embryos at a

concentration of 250 μg/mL, as well as decreased human osteosarcoma U2OS cell expression of Vascular endothelial growth factor (VEGF), angiopoietin-2, bFGF, Platelet-derived growth factor (PDGF), and transforming growth factor (TGF)-β-1 [15]. Though the results from the cellular proliferation study were questionable due to the large difference between the effect at 500 and 1000 μg/mL, it is only one of the parameters involved in tumor growth, as indicated above. Furthermore, studies in progress indicate that NM causes increased apoptosis in lung cancer A549 cells with higher doses, especially at NM 500 and 1000 μg/mL. In addition, a study on the effects of this nutrient mixture on human T-lymphocytic virus (HTLV)-1–positive (HuT-102 and C9-PL) and–negative (CEM and Jurkat) cells revealed that this combination of nutrients significantly increased apoptotic cells in the preG$_1$ phase, secondary to up-regulation of p53, p21, and Bax protein levels and down-regulation of Bcl-2α protein expression [16].

Control of proteolytic activity of ECM provides an opportunity of addressing common mechanism of metastasis, angiogenesis and tumor growth. Research has shown that highly metastatic lung cancer and other cancer cells secrete higher amounts of MMPs than do poorly metastatic cells. Therefore, prevention of ECM degradation through the inhibition of MMP activity, specifically MMP-2 (gelatinase A) and MMP-9 (gelatinase B) has been shown to be a promising therapeutic target in blocking tumor progression. Rath and Pauling postulated [12] that cancer growth and metastasis can be controlled through a natural approach, targeting the stability of the connective tissue surrounding cancer and therefore contributing to tumor "encapsulating" effect, using nutritional components, such as lysine and lysine analogues. Lysine interferes with the 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 [12]. A recent study demonstrated 7-fold reduction in metastasis of mammary cancer in plasmin deficient transgenic mice [25]. 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 upon 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.

As mentioned previously, MMPs, especially MMP-2 and MMP-9, are also involved in tumor metastasis. Currently there is no effective treatment to blocking lung metastasis, and discovery of antimetastatic agents, especially nontoxic ones, would dramatically improve lung cancer prognosis. Invasion of host tissues is a hallmark of metastasis, a process dependent on tumor

cell adhesion, cell migration, and proteolytic degradation of the ECM by MMPs [26]. The results from our in vitro study of human lung cancer cells demonstrated potent, significant suppression of MMP secretion and Matrigel invasion and migration of A-549 cells at 500 μg/mL. These results corroborate with our earlier data obtained with various types of cancer cell lines, indicating that NM affects mechanisms involved in cellular invasion that are universal for various types of cancers rather than being cancer specific [13–16].

The inhibitory effects of the individual nutrients tested have been reported in both clinical and experimental studies. Ascorbic acid has been reported to exert cytotoxic and antimetastatic actions on malignant cell lines [21, 27–30]; in addition, low levels of ascorbic acid have been reported in cancer patients [31, 32]. Green tea extract is a potent anticancer agent that has been reported to have antimetastatic and anti-angiogenic effects against human cancer cell lines; however, its effects were observed at much higher doses than available in food sources [33–35]. In this aspect, our previous studies indicated that the inhibitory 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, allowing for obtaining maximum effects using lower doses of individual components [24].

Over the years, cancer therapy has witnessed many exciting developments, but a cure for cancer still remains to be found, because therapeutic approaches have relied mainly on killing tumors, without fully realizing the mechanisms controlling cancer cell death. Shifting research directions towards controlling cancer cell invasiveness in the tissues and their growth opens up a possibility of finding effective controls of this disease. Application of natural components in mixtures, not individually, can assure enhanced effectiveness on multiple targets.

Furthermore, in contrast to the toxic side effects of current mono and doublet chemotherapy, the nutrient mixture has been shown to be safe therapeutic agent. In a previous in vivo study addressing safety issues, we found that gavaging adult female ODS rats (weighing 250 to 300 g) with the nutrient mixture (at 30, 90, or 150 mg per day for 7 days), had neither adverse effects on vital organs (heart, liver, and kidney), nor on the associated functional serum enzymes, indicating that this mixture is safe to use even at these high doses, which far exceed the normal equivalent dosage of the nutrient [36]. Specifically, no difference was observed in liver serum levels of protein, albumin (A), globulin (G), A/G ratio, alkaline phosphatase, and aspartate aminotransferase (AST) between treated and control groups. However, alanine aminotransferase slowly decreased with increased dose of NM. In regard to heart serum levels, lactate dehydrogenase, creatine kinase, and AST in test groups did not differ from control groups. In regard to renal function, creatinine content (C) was the same for the test

and control groups, and blood urea nitrogen (BUN) (B) showed a decrease, as did the B/C ratio, with an increased concentration of NM. Furthermore, in regard to lipid levels, in the NM groups, both cholesterol and triglyceride levels decreased compared to control groups. There was no difference in the histopathology of liver, kidney, heart, and lung compared to control groups and amongst test groups.

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Chemopreventive effect of a novel nutrient mixture on lung tumorigenesis induced by urethane in male A/J mice

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ABSTRACT

Aims and background. Lung cancer, a leading cause of cancer death, is associated with exposure to inhalation carcinogens, most commonly those found in tobacco smoke. We investigated the *in vivo* effect of dietary supplementation with a nutrient mixture containing lysine, proline, arginine, ascorbic acid, green tea extract, N-acetyl cysteine, selenium, copper and manganese on the development of urethane-induced lung tumors in male A/J mice.

Methods. After one week of isolation, seven-week-old male A/J mice (n = 25) weighing 17-19 g were randomly divided into three groups: group A (n = 5), group B (n = 10), and group C (n = 10). Mice in groups B and C were each given a single intraperitoneal injection of urethane (1 mg/g body weight) in saline, whereas group A mice received an injection of saline alone. Groups A and B were fed a regular diet, whereas group C was fed the same diet supplemented with 0.5% nutrient mixture. After 20 weeks, mice were sacrificed, lungs were excised and weighed, and tumors were counted and processed for histology.

Results. Urethane-challenged mice developed tumors. However, the mean number of tumors and the mean lung weights in the mice on the supplemented diet were significantly reduced, by 49% ($P < 0.0001$) and 18% ($P = 0.0025$), respectively, compared to mice on the control diet. We observed neither significant differences in body weight gains nor in diet consumption among the mice. Pulmonary lesions were morphologically similar for both the groups (adenomas), but lesions were smaller in the test group.

Conclusions. The results suggest that nutrient mixture has inhibitory potential on the development of mouse lung tumors induced by urethane

Introduction

Lung cancer is the leading cause of cancer-related death for men and women worldwide, and the second most common cancer among men and women in the USA[1]. Lung cancer mortality among women has continued to increase slightly whereas it has stabilized among men[2]. The greatest risk factor for developing lung cancer is exposure to inhalation carcinogens, most commonly those found in tobacco smoke. Cigarettes smoked per year reached 5.6 million at the close of the twentieth century, and it is predicted to cause nearly 10 million deaths by the year 2030[3]. Approximately 90% of male lung cancers and 70% of female lung cancers are attributed to smoking[4]. Other risk factors include asbestos and radon exposure. Despite improvements in therapy, the cure rate for lung cancer remains low. In general, surgery, radiation and chemotherapy are used in the management of lung cancer. However, these treatments have adverse and toxic effects.

In humans, most primary lung tumors have metastasized by initial clinical diagnosis. Due to lack of noninvasive scanning procedures, such as endoscopy used for

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the detection of colon cancer, detection of early events leading to the pathologies characteristic of lung cancer is difficult. In contrast, murine tumors provide an excellent tool for observation of the effect of therapeutic agents on a timed multistage process leading to malignancy[5]. Malkinson[5] reported the following time scale of multistage carcinogenesis in mice after application of a carcinogen: hyperplasia at 2 weeks, microscopic benign tumors at 4 weeks, grossly visible benign tumors at 10 weeks, medium to large benign tumors at a few months, adenocarcinoma after 6 months, and metastasis at a year.

We chose to study the urethane-induced lung carcinogenesis model in A/J mice, since the most common form of lung cancer diagnosed worldwide is adenocarcinoma, and murine adenocarcinoma has molecular, histologic and morphologic similarities to human adenocarcinoma. The urethane-induced lung carcinogenesis model in A/J mice is frequently employed in chemoprevention studies[6].

Nutrients have been shown to inhibit lung carcinogenesis in animal models. For example, Mimoto *et al.*[7] found that epigallocatechin gallate treatment (1 mg/ml in tap water) administered 2 weeks prior to cisplatin administration (1.62 mg/kg body wt, i.p.) significantly reduced lung tumorigenesis in A/J mice. Black and green teas were reported to prevent lung cancer induced by tobacco-specific carcinogen in rodents[8]. Singh *et al.*[6] found that silibinin, the flavanone from milk thistle, inhibited lung tumor angiogenesis in A/J mice. We have developed strategies to inhibit cancer development and its spread using a mixture of naturally occurring nutrients such as lysine, proline, ascorbic acid and green tea extract (nutrient mixture, NM), which have exhibited anticancer activity *in vivo* and *in vitro* in a number of cancer cell lines, including human and murine melanoma cells lines[9-11], inhibiting cancer cell growth, matrix metalloproteinase secretion, invasion, metastasis, and angiogenesis. The present study examines the *in vivo* effect of NM in the development of urethane-induced lung tumors in male A/J mice.

The NM was formulated based on targeting different physiological processes involved in cancer progression and metastasis at the cellular level. Natural inhibitors of extracellular matrix proteolysis, such as lysine, proline and ascorbic acid are essential for formation and optimum stability of collagen fibers that comprise the extracellular matrix. Manganese and copper are also essential cofactors in collagen formation process. Collagen stability is also modulated by N-acetyl cysteine through inhibition of matrix metalloproteinase activity[12] and tumor cell invasion[13]. Selenium has been shown to interfere with migration of endothelial cells through the extracellular matrix[13], as well as to inhibit MMP expression and tumor invasion[14]. In addition to its critical role in extracellular mixture structure, ascorbic acid has been shown to inhibit cancer cell division and growth through production of hydrogen peroxide, especially in high doses[15,16]. Green tea extract has been shown to be a promising agent in controlling tumor growth, metastasis, and angiogenesis[17]. Arginine, a precursor of nitric oxide (NO), acts predominantly as an inducer of apoptosis[18].

Materials and methods

Animals

Male A/J mice, free of murine viruses, bacteria and parasites, approximately 6 weeks of age on arrival, were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) and maintained in microisolator 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.

Materials

Urethane was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Regular mouse chow and supplemented mouse chow were milled and pressed by Purina Mills (St. Louis, MO, USA). The NM, which was prepared by VitaTech (Hayward, CA, USA), consisted of the following ingredients in the relative amounts indicated: 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 (80% polyphenol), 1000 mg; selenium, 30 μg; copper, 2 mg; manganese, 1 mg. Mice consumed approximately 4 g of chow per day.

Experimental design

After one week of isolation, 7-week-old male A/J mice (n = 25) weighing 17-19 g were randomly divided into three groups: group A (n = 5), group B (n = 10), and group C (n = 10). Groups B and C mice each received a single i.p. injection of urethane (1 mg/g body weight) in saline, whereas group A mice received an injection of saline alone. The urethane dose, animal model and time of study chosen were based on the work of Singh *et al.*[6] and the tumor staging diagram of Malkinson[5]. Tumor developmental stages in A/J mice post urethane injection are as follows: lung hyperplasia in 2-3 weeks, microscopically visible lung adenomas in approximately 5 weeks, and macroscopically visible lung adenomas (30-50) in approximately 15 weeks[6]. Death due to respiratory distress caused by the massive increased tumor burden typically begins at 40 weeks after urethane injection[5]. Groups A and B were fed a regular diet, whereas group C was fed the same diet supplemented with 0.5% NM. After 20 weeks, mice were sacrificed, the lungs were excised and weighed, and tumors were counted and processed for histology.

Histology

Tissue samples were fixed in 10% buffered formalin. All tissues were embedded in paraffin and cut at 4-5 microns. Sections were deparaffinized through xylene and graduated alcohol series to water and stained with hematoxylin and eosin for evaluation using a standard light microscope.

Statistical analysis

The results were expressed as means ± SD. Data was analyzed by the independent sample *t* test. The D'Agostino-Pearson test for normal distribution was used to analyze the initial and final weights of the groups, and Pearson's correlation coefficient was determined for lung weight and tumor multiplicity correlation, using MedCalc Software (Mariakerke, Belgium).

Results

Mean body weights

We observed neither significant differences in body weight gains nor in diet consumption among the mice. Initial mean body weights of mice ranged between 17 and 19 g (17.8 ± 1.6, which met the D'Agostino-Pearson test for normal distribution) and final weights ranged between 28 and 30 g (27.8 ± 2.2, which met the D'Agostino-Pearson test for normal distribution). Lack of a significant difference in body weight and dietary consumption among the mice indicated that the observed decrease in lung weights in group C compared to that in group B was not due to caloric restriction.

Tumor multiplicity

Mice in both urethane-injected groups developed tumors in the lung, but the mice supplemented with 0.5% NM demonstrated a significantly reduced mean number of tumors (by 49%, $P<0.0001$) compared to the control group of mice, as shown in Figures 1 and 2. Mice injected with the vehicle alone did not develop lung tumors. No tumors were evident in the liver, kidney, spleen, or heart in any of the mice.

Organ weight

Mean lung weight of animals from the urethane-treated control group was 44% ($P = 0.0002$) higher than that of the untreated control. Among urethane-treated mice, NM supplementation resulted in an 18% ($P = 0.0025$) reduction in mean lung weight compared to the control diet. Mean liver weights were also affected. Urethane treatment increased mean weight by 23% ($P = 0.02$) over the untreated control, and NM supplementation reduced the effect of urethane administration by 12% ($P = 0.02$). However, no tumors were evident in the liver. No metastasis is seen at this stage of carcinogenesis of primary lung tumors. Kidney, spleen and heart mean weights did not differ significantly among the groups (Table 1).

Correlation between lung weight and tumor multiplicity in AJ mice

To determine whether there was a relationship between tumor multiplicity and lung weight, data from all mice in the study were pooled. In analyzing the data, a significant positive correlation was obtained (correlation coefficient r = 0.7891, $P<0.0001$), as shown in Figure 3.

Histopathology

Pulmonary lesions were morphologically similar for both the groups (adenomas), but lesions were smaller in the NM-supplemented group. Histological examination revealed adenomas were characterized by well-differentiated cuboidal cells with irregular round nuclei and clear to slightly eosinophilic cytoplasm, forming glandular or papillary structures (Figures 4 and 5).

Discussion

The results of the study demonstrated that chronic oral consumption of the NM by A/J male mice exposed to the carcinogen urethane reduced the development and growth of primary lung tumors. Benign lung tumors were observed in both urethane-injected groups, ranging between a mean number of tumors per mouse of 39 in the urethane-treated control and 20 in the urethane-challenged mice supplemented with 0.5% NM (a significant reduction in mean number of tumors, by 49%, $P<0.0001$).

In addition, not only was there reduced tumor multiplicity in the NM-supplemented mice, but it was also noted that the tumors were smaller in the NM-supplemented mice than in the control-diet mice. At 20 weeks, the tumors were benign but visible, as predicted by the staging diagram of Malkinson[5]. Among urethane-treated mice, NM supplementation resulted in an 18%

Table 1 - Effect of urethane administration and NM supplementation on mean organ weights (g) of A/J mice

Organ	Mean organ weight (g)		
	Group A (negative control)	Group B (urethane-treated with control diet)	Group C (urethane-treated with 0.5% NM diet)
Lung	0.16 ± 0.01	0.23 ± 0.03	0.19 ± 0.02
Liver	1.02 ± 0.16	1.25 ± 0.15	1.1 ± 0.11
Kidney	0.35 ± 0.07	0.38 ± 0.05	0.36 ± 0.04
Heart	0.10 ± 0.01	0.11 ± 0.01	0.10 ± 0.04
Spleen	0.09 ± 0.03	0.08 ± 0.02	0.07 ± 0.02

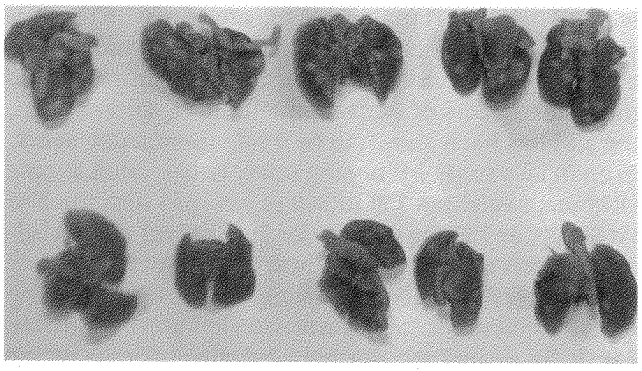

Figure 1 - Representative gross lung specimens of urethane-treated A/J mice. NM-supplemented (group C), shown in the bottom row, showed fewer tumors than did control diet (group B), shown in the top row of lung specimens.

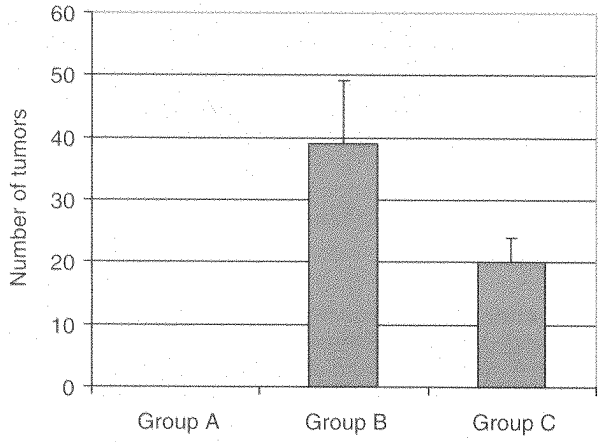

Figure 2 - Effect of supplementation with 0.5% NM on mean number of lung tumors per group. Among urethane-injected mice, NM supplementation (group C) significantly reduced multiplicity by 49% (P <0.0001) compared to consumption of the control diet (group B). No tumors were evident in the negative control group (group A).

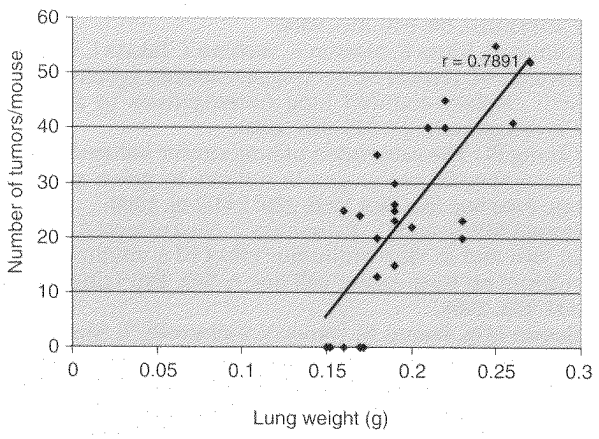

Figure 3 - Correlation between tumor multiplicity and lung weight. Analysis of pooled data from the study revealed a significant positive correlation (r = 0.7891, P <0.0001) between lung tumor multiplicity and lung weight.

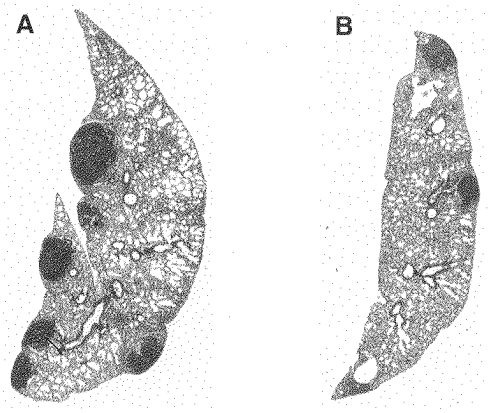

Figure 4 - Representative lung sections from urethane-treated A/J mice. Fewer and generally smaller lesions were evident in the NM-supplemented mice (group C) than in the control diet mice (group B). A) Control diet group. B) 0.5% NM-supplemented diet group.

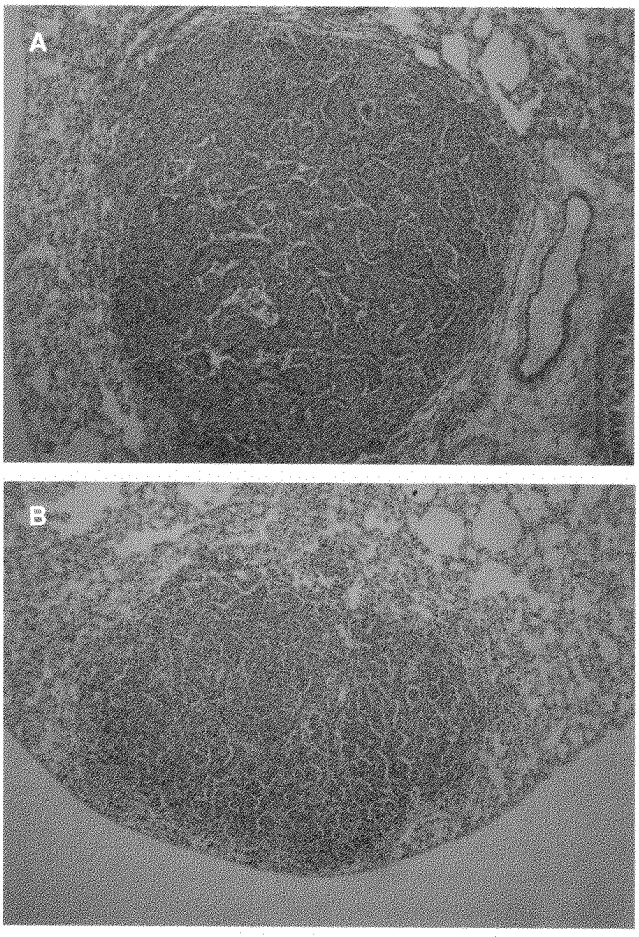

Figure 5 - Histopathology of urethane-induced tumors in A/J mice. Histological evaluation revealed adenomas in both groups, characterized by well-differentiated cuboidal cells with irregular round nuclei and clear to lightly eosinophilic cytoplasm forming glandular or papillary structures. A) Control diet group. B) 0.5% NM-supplemented diet group.

($P = 0.0025$) reduction in mean lung weight compared to the control diet. Since there was no significant difference in mean body weights of mice, these findings indicate that the observed difference in mean lung weight among the groups of mice is due to differences in tumor multiplicity and tumor growth. As mentioned in the Results section, to determine whether there was a relationship between tumor multiplicity and lung weight, we pooled data from all mice in the study and compared these parameters. In analyzing the data, a significant positive correlation was obtained (correlation coefficient r = 0.7891, $P < 0.0001$) between tumor multiplicity and lung weight.

As discussed earlier, all organs were observed visually and found to be free of tumors, except the lungs. Mean weights of the kidney, heart, and spleen did not differ significantly among all the groups, as expected. However, mean liver weight was higher in the urethane-treated control than in the negative control and in the NM-supplemented group. The difference was not due to tumor incidence in the liver, as metastasis had not occurred. Urethane-associated hepatic toxicity may have caused the increased liver weight, which was inhibited by NM supplementation. In measuring the urethane level in various organs of mice, the levels in livers of mice challenged with urethane had been shown to be relatively high[19]. Cytochrome P450 2E1 has been shown to be the principal enzyme responsible for urethane metabolism, leading to formation of vinyl carbamate epoxide, the ultimate carcinogen binding to macromolecules DNA, RNA and proteins[19,20]. In a previous study, we found that acetaminophen hepatic toxicity, based on histopathology and functional serum enzymes, was reduced by dietary supplementation with 0.5% NM compared to mice on the control diet[21].

Tumor growth depends on the generation of new blood vessels to sustain survival and proliferation. Growing tumors become hypoxic and acidotic beyond the size of 2 mm and secrete several growth factors to stimulate local blood vessels to sprout branches. Activation of endothelial cells gives rise to production of matrix metalloproteinases, allowing for the decomposition of the surrounding extracellular matrix and eventual development of new blood vessels. Our previous studies demonstrated significant antitumor effects of NM in a number of cancer cell lines *in vivo* and *in vitro*[9,10,22]. Composition of the NM was identical in all cited studies. The NM has also exhibited strong antiangiogenic properties, using models such as the chorioallantoic membrane assay in chick embryos and basic fibroblast factor-induced vessel growth in C57BL/6 mice[11]. Circulating levels of vascular endothelial growth factor and basic fibroblast factor are statistically higher in non-small-cell lung cancer patients than in healthy control subjects, and the extent of response to chemotherapy is associated with the level of vascular endothelial growth factor[23].

In conclusion, the results of the present study demonstrate that chronic consumption of NM-supplemented diet by A/J male mice injected i.p. with the carcinogen urethane inhibited the multiplicity and growth of primary lung tumors. Furthermore, in contrast to the toxic side effects of current chemotherapeutic agents, the NM has been shown to be a safe therapeutic agent. In an *in vivo* study addressing safety issues, adult female ODS rats (weighing 250 to 300 g) gavaged with the NM (at 30, 90, or 150 mg per day for 7 days) had neither adverse effects on vital organs (heart, liver and kidney) nor on the associated functional serum enzymes, indicating that the mixture is safe to use even at such high doses, which far exceed the normal equivalent dosage of the nutrient[24].

The present study was conducted on mice, and although the animal model mirrors human lung cancer, there is the usual limitation regarding translation of an animal model to practical use in humans. Additionally, it is not known which mechanisms, such as induction of apoptosis, inhibition of matrix proteolysis, or detoxification, are responsible for the effect of NM on the growth of the urethane-induced primary tumor. We are currently investigating these mechanisms in greater detail.

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