

Suppression of Matrix Metalloproteinases -2 and -9 in Various Human Cancer Cell Lines by a Nutrient Mixture

Keywords: MMP-2; MMP-9; Cancer biomarkers; Metastasis

Abstract

The matrix metalloproteinases (MMPs) are a family of zinc containing endopeptidases that degrade various components of the extra cellular matrix. Among the many types of MMPs that have been identified, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are thought to play a key role in cancer metastasis. These MMPs are able to modify tumor microenvironment by degrading type IV collagen found in the cellular basement membrane. MMP-2 and -9 are essential in facilitating cancer cell invasion, tumor progression, and metastasis, thereby shortening patient survival in all cancer types. A significant association has been reported between tumor aggression and increased levels of MMP-2 and MMP-9. Therefore, MMPs are used as diagnostic and prognostic biomarkers in many clinical trials and experimental studies. MMP inhibitors (MMPIs) seem to be the logical targets for the therapeutic intervention in cancer. The rationale for developing MMPIs for halting cancer has been around for more than three decades. However, the clinical trials of synthetic MMPIs have not produced favorable results.

In this review, we summarize the results of our *in vitro* studies evaluating effects of a natural and non-toxic nutrient mixture on inhibition of the cancer biomarkers; MMP-2 and MMP-9 in the treatment and control of metastasis of 42 different cancer cell lines from 13 representative classes of malignancies.

Abbreviations

MMP: Matrix Metalloproteinases; NM: Nutrient Mixture; ECM: Extracellular Matrix; PMA: Phorbol 12-Myristate 13-Acetate; ER: Estrogen Receptor; EGCG: Epigallocatechin Gallate

Introduction

Cancer is the second leading cause of death in the world, striking people of all ages [1-3]. Yearly cancer deaths are projected to increase to over 11 million deaths worldwide by 2030. On an individual level, the overall risk of developing cancer during one's lifetime is 50% for men and 33% for women [1,2]. The most frequent cancers in men are lung, prostate, and colorectal cancer, in that order, while in women, the cancers with highest incidence are breast, cervical, and colorectal cancer. The highest mortality is associated with lung, stomach, and liver cancer [1-4].

The treatment regimen used for a specific cancer depends primarily on the type of cancer and the stage of cancer. For most cancers, surgery, radiotherapy, and chemotherapy are preferred treatment modalities. However, all three options are associated with several short and long-term detrimental side effects for the patient [4,5]. Chemotherapy and radiotherapy indiscriminately attack



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healthy as well as cancer cells, causing extensive damage throughout the body. Moreover, analysis of the clinical trials from 1990 to 2004 on 22 types of cancers showed that chemotherapeutic intervention increases the five-year survival rate by a mere 2.1% [1-4]. Currently, researchers are exploring targeted compounds for cancer treatments to reduce wide spread adverse effects.

Cancer cells secrete zinc-dependent endopeptidases, the matrix metalloproteinases (MMPs), which remodel tumor's surrounding microenvironment by cleaving collagen fibers and other extracellular matrix components, thereby facilitating metastasis [6-18]. More than 90% of the cancer deaths are associated with metastatic cancer [4-10].

Therefore, finding a safer and effective alternative to inhibit the action of MMP enzymes would potentially avoid side effects and damage to the healthy cells, while treating cancer patients.

The steps of the metastatic process include detachment of cancer cells from the primary tumor, disruption of the basement membrane, invasion into the surrounding stroma, cancer cell entry into and transport through the vascular or lymphatic system, cancer cell colonization, angiogenesis, and proliferation. Each step of metastasis is initiated and governed by one or more of the 25 known MMP enzymes. Additionally, MMP enzymes are also have prognostic significance as cancer biomarkers to indicate aggressiveness and metastatic potential.

Although approximately 25 different MMPs act on a broad spectrum of substrates, including collagen type I, II, III, and IV, and stromyelin, of these MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are particularly attractive targets of MMP-2 and MMP-9 inhibitors (MMPI) research because of their central role in tumor metastasis [5-15]. MMP-2 and MMP-9 play prominent roles in the degradation of type IV collagen found in the basement membrane and extracellular matrix [16-21]. MMP-2 and MMP-9 have the ability to modify the tumor microenvironment, a complex extracellular matrix network that mediates interactions between tumor cells, stromal cells, and immune cells. Alterations in the tumor microenvironment can cause changes in local metabolism, tumor growth, tumor progression, treatment resistance, and, eventually, metastatic potential. Indeed, a significant association has been reported between both experimental and clinical tumor aggression and increased levels of MMP-2 and MMP-9. Thus, the challenge is to develop an effective MMP inhibitor

that utilizes minimally toxic and readily absorbed naturally derived substances, which slow or inhibit MMP-2 and MMP-9 action.

Numerous studies have found that certain naturally derived substances individually exhibit potent inhibitory effects on the progression of a wide variety of cancers [22-43]. The NM is a combination of several nutrients formulated to target the key physiological pathways in cancer progression and metastasis. 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 [29] and by N-acetyl cysteine (NAC) through its inhibitory effect on MMP-9 activity [30] and invasiveness of tumor cells [31]. Ascorbic acid, among other anti cancer actions, is also proven to inhibit cancer cell division and growth through production of hydrogen peroxide [32-37]. Green tea extract is known to be a promising agent in controlling angiogenesis, metastasis, and other aspects of cancer progression [38,39]. In addition, selenium has been shown to interfere with MMP expression and tumor invasion and to induce selective apoptosis of cancer cells [40,41]. Since, arginine is a precursor of nitric oxide (NO); any deficiency of arginine can limit the production of NO, which predominantly acts as an inducer of apoptosis [42]. Furthermore, we have reported that the effects of a specific combination of these nutrients were superior to their individual effects or their random combination on anti- proliferative action on cancer cells [43].

The research from our group has also shown that combining these naturally derived substances achieves a synergistic effect against MMP-2 and MMP-9 secretion [44-49]. This synergistic nutrient mixture (NM) used in this review is a combination of several nutrients formulated to target the key physiological pathways in cancer progression and metastasis.

We studied the effect of NM on the secretion of MMP-2 and MMP-9 by 42 cancer cell lines using gelatinase zymography (Table 1, Figure 1). The cancer cell lines studied were grouped by organ systems such as female breast and gynecological cancers, testicular, prostate and male breast cancers, gastrointestinal cancers, lung cancer, mesothelioma, head and neck cancers, pediatric sarcomas, adult sarcomas, leukemias, and others, to determine the therapeutic potential of NM in preventing metastases of these cancers. Figure 1 demonstrates the secretion of MMP-2 and MMP-9 in melanoma cell lines A 2058, with and without PMA (100 ng/ml).

Methods and Materials

Cancer cell lines and reagents

42 different cancer cell lines were selected based on different patterns of MMP-2 and MMP-9 secretion, and included various carcinomas, adenomas, soft tissue sarcomas, and leukemias (Table 1). The cancer cell lines and their recommended media were purchased from ATCC (Rockville, MD, USA). All other reagents, including fetal bovine serum (FBS), penicillin, streptomycin, phorbol 12-myristate 13-acetate (PMA), were of high grade and obtained from Sigma (St. Louis, MO). All other reagents used were of high purity and were obtained from Sigma, unless otherwise indicated.

Composition of the nutrient mixture (NM)

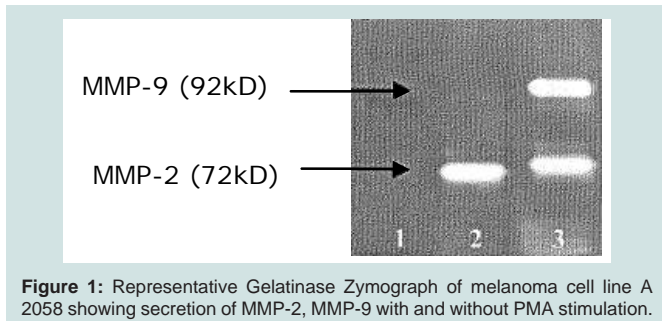
The nutrient mixture (NM) includes the following: Vitamin C (as ascorbic acid and as Mg, Ca and palmitate ascorbate) 710 mg; L-lysine 1000 mg; L-Proline 750 mg; L-Arginine 500 mg; N-Acetyl Cysteine 200 mg; Standardized Green Tea Extract (80% polyphenol) 1000 mg; Selenium 30 µg; Copper 2 mg; and Manganese 1 mg.

Cell culture

Cancer cell lines were grown in recommended media, supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 µg/mL) in 24-well tissue culture plates (Coster,

Table 1: Cancer cell lines classified by system malignancy.

<p>Male Cancers</p> <p>Prostate: DU-145, PC-3, LNCAP Testis: NTER-2 Male Breast Cancer: Colo-824</p> <p>Female Cancers</p> <p>Breast: MDA-MB-231, MCF-7 Cervix: HeLa, DoTc2-4510 Ovary: SK-OV-3 Uterus: SK-TU-1, MES-SA, MESSA/ DX5</p>	<p>Respiratory</p> <p>Lung: A-549 Mesothelioma: MSTO-211H</p> <p>Gastrointestinal</p> <p>Colon: HCT-116 Liver: Sk-Hep-1, Hep-G2 Pancreas: MiaPaCa-1</p> <p>Reno-Uriinary</p> <p>Kidney: RCC-786-0 Bladder: T-24</p>	<p>Adult Sarcomas</p> <p>Chondrosarcoma: SW-1353 Fibrosarcoma: HT-1080 Liposarcoma: SW-872 Synovial Sarcoma: SW-982</p> <p>Pediatric Sarcomas</p> <p>Osteosarcoma: MNNG-HOS, SK-ES-1, U-2OS Rhabdomyosarcoma: RD (Embryonic)</p> <p>Leukemias:</p> <p>HL-60, Jurkat, Raji</p>	<p>Head and Neck Cancers</p> <p>Fanconi associated head and neck cancer: OHSU-974 Laryngo-pharyngeal: FaDu Tongue: SCC-25 Thyroid: SW579</p> <p>Nervous System</p> <p>Neuroblastoma: SK-N-MC Retinoblastoma: Y-79 Glioblastoma: A-172, LN-18, T-98G</p> <p>Melanoma A-2058</p>
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Cambridge, MA, USA). The cells were plated at a density of 1×10^5 cells/mL and grown to confluence in a humidified atmosphere at 5% CO₂ at 37 °C. At near confluence, serum-supplemented media were removed and the cell monolayer was washed once with PBS and with the recommended serum-free medium. The cells were then treated with the nutrient mixture (NM), dissolved in media, and tested at 0, 10, 50, 100, 500, and 1000 µg/mL in triplicate at each dose. Parallel sets of cultures were treated with PMA (100 ng/ml) for induction of MMP-9. The plates were then returned to the incubator. The conditioned media were collected separately, pooled, and centrifuged at 4 °C for 10 min at 3000 rpm to remove cells and cell debris. After 24 hours, the media were removed and the supernatant was collected and used for gelatinase zymography, which is a highly sensitive assay of gelatinolytic enzymatic activity able to detect both pro and active forms of MMP-2 and MMP-9.

Gelatinase zymography

Gelatinase zymography was performed in 10% NOVEX Pre-Cast SDS Polyacrylamide Gel (Invitrogen Corporation) in the presence of 0.1% gelatin under non-reducing conditions. Culture media (20 µL) were mixed with sample buffer and loaded for SDA-PAGE with *tris*-glycine SDS buffer as suggested 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 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 Brilliant Blue R250 in 50% methanol and 10% glacial acetic acid for 30 min and destained. Upon renaturation of the enzyme, the gelatinases digest the gelatin in the gel and give clear bands against an intensely stained background. Protein standards were run concurrently and approximate molecular weights were determined by plotting the relative mobilities of known proteins. Gelatinase zymograms were scanned using a CanoScan 9950F Canon scanner at 300 dpi. The intensity of the bands was evaluated using the pixel-based densitometer program Un-Scan-It, Version 5.1, 32-bit, by Silk Scientific Corporation (P.O. Box 533, Orem, UT 84059, USA), at a resolution of 1 Scanner Unit (1/100 of an inch for an image that was scanned at 100 dpi). The pixel densitometer calculates the optical density of each pixel (values 0 to 255) using the darkly stained background of the gel as a pixel value of zero. A logarithmic optical density scale was used since the optical density of films and gels is logarithmically proportional to the concentration. The pixel densitometer sums the optical density of each pixel to give a band's density. In all graphs, band densities were reported as percentages of the sums of all pixels in a given lane of a gel.

The effect of the NM on MMP secretion was assessed by treating cells to increasing concentrations of NM and expressed as relative band densities (as % of control value). The cumulative effect of NM was determined by summing all the band densities within a treatment group and comparing these densities between treatment groups. The extent of total MMP band secretion in a treatment group was reported as 'total band pixel'.

Results

Female cancers (breast and other gynecological cancers)

Breast cancer: It is the most common cancer diagnosed in women and is the second leading cause of deaths, after lung cancer, in women in the world. Breast cancer, when diagnosed early, can be treated using surgery, radiation, chemotherapy, or hormonal therapy. There is no complete cure and the 5-year survival of all patients with breast cancer is 88% when given appropriate treatment [1-4,52,50].

We investigated the effect of NM on estrogen receptor (ER) positive and ER negative breast cancer cell lines; MCF-7 and MDA-MB-231, respectively. Neither of the cell lines showed any secretion of MMP-2 even after stimulation by PMA. However, both cell lines secreted MMP-9 upon PMA stimulation. NM treatment resulted in a dose-dependent suppression of MMP-9 secretion in both cell lines. Exposure of ER negative MDA-MB-231 at 50 µg/mL NM concentration resulted in a complete suppression of MMP-9 secretion and in the MCF-7 the secretion of MMP-9 was inhibited at 500 µg/mL NM (Table 2).

Cervical cancer: It is the second most common cancer of the female reproductive tract and is currently the third leading cause of cancer death in women. More than 85% of deaths due to cervical cancer occur in developing countries and the reduced mortality in the developed countries is mostly attributed to regular pap smear testing. The incidence of cervical cancer is highest among women with a history of sexually transmitted diseases [1-4].

We investigated the effect of NM on cervical cancer cell line, HeLa, which secreted MMP-2 and MMP-9, with and without PMA stimulation. The NM decreased levels of both enzymes in a dose-dependent manner both in the presence and in absence of PMA. A complete inhibition of MMP-2 secretion occurred at 500 µg/mL NM and MMP-9 at 10 µg/mL NM (Table 2; Figures 2A and 2B). Cervical cancer cell line DoTc2-4510 secreted only MMP-9, with responded to PMA stimulation. NM inhibited MMP-9 secretion in a dose-dependent manner, with its complete inhibition at NM concentration of 500 µg/ml.

Ovarian cancer: This is the deadliest of the gynecological cancers, and is the fifth leading cause of cancer death among US women. The lethal nature of ovarian cancer is due to its ability to metastasize rapidly. In most cases, the cancer is already advanced at the time of diagnosis. Currently, 50% of the women diagnosed with ovarian cancer die from that cancer within five years [1-4,50].

We investigated the NM effect *in vitro* on the human ovarian cancer cell line SK-OV-3, which secreted MMP-2 after PMA stimulation. NM inhibited MMP-2 secretion in a dose-dependent manner with its complete inhibition at 50 µg/mL NM concentration (Table 2).

Table 2: Effect of nutrient mixture on MMP-2 and MMP-9 expression in female cancers.

		MMP-2						MMP-9					
		0 mg/mL	10 mg/mL	50 mg/mL	100 mg/mL	500 mg/mL	1000 mg/mL	0 mg/mL	10 mg/mL	50 mg/mL	100 mg/mL	500 mg/mL	1000 mg/mL
1. Breast Cancers													
i) MDA-MB-231	No PMA	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	w/PMA	0%	0%	0%	0%	0%	0%	100%	14%	0%	0%	0%	0%
ii) MCF-7	No PMA	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	w/PMA	0%	0%	0%	0%	0%	0%	100%	100%	80%	10%	2%	1%
2. Gynecological Cancers													
Cervical Cancer													
i) (HeLa)	No PMA	100%	120%	70%	8%	0%	0%	100%	0%	0%	0%	0%	0%
	w/PMA	100%	94%	84%	74%	16%	0%	100%	96%	76%	33%	0%	0%
ii) (DoTc2-4510)	No PMA	0%	0%	0%	0%	0%	0%	100%	67%	12%	5%	0%	0%
	w/PMA	0%	0%	0%	0%	0%	0%	100%	41%	22%	1%	1%	0%
Ovarian Cancer													
i) (SK-OV-3)	No PMA	100%	40%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	w/PMA	100%	39%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Uterine Sarcomas													
i.) SK-UT-1	No PMA	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	w/PMA	0%	0%	0%	0%	0%	0%	100%	90%	73%	13%	3%	0%
ii.) MES-SA	No PMA	100%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	w/PMA	100%	18.3%	0%	0%	0%	0%	100%	70.5%	47.4%	0%	0%	0%
iii.) MES-SA/ DX5	No PMA	100%	31.4%	9.8%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	w/PMA	100%	30.2%	0%	0%	0%	0%	100%	76.3%	0%	0%	0%	0%

Uterine cancer: Unlike cervical cancer, uterine cancer is twice as common in developed countries. Uterine sarcomas are a rare type of cancer that accounts for 5% of all uterine malignancies. The most common type is a carcinosarcoma arising from the endometrium and from the muscle cells. The second most common accounting for 30% of all uterine sarcomas is a leiomyosarcoma, which has a peak incidence at age 50. Sarcomas arising from the stroma of the endometrium account for 15% of all uterine sarcomas.

We investigated the *in vitro* effect of NM on the leiomyosarcoma cell line SK-UT-1, the uterine sarcoma cell line MES-SA, and on multi-drug resistant uterine sarcoma cell line MES-SA/DX5. SK-UT-1 did not secrete either MMP-2 or MMP-9 constitutively. MMP-9 secretion

in this cell line was induced after PMA treatment, both, active and inactive forms. This cell line exposed to NM showed dose-dependent inhibition of MMP-9 secretion with complete inhibition at 1000 µg/ml. MES-SA and MES-SA/DX5 secreted only MMP-2 constitutively. MMP-9 secretion required stimulation by PMA. Secretion of MMP-2 and MMP-9 by MES-SA and MES-SA/DX5 cells, both before and after treatment with PMA, was inhibited by NM in a dose-dependent manner with complete suppression occurring at a NM concentration of 10-100 µg/ml (Table 2).

Male cancers (prostate, testicular, male breast cancer)

Prostate cancer: Prostate cancer is the most common cancer in men and second most deadly cancer in the US [1-4], primarily

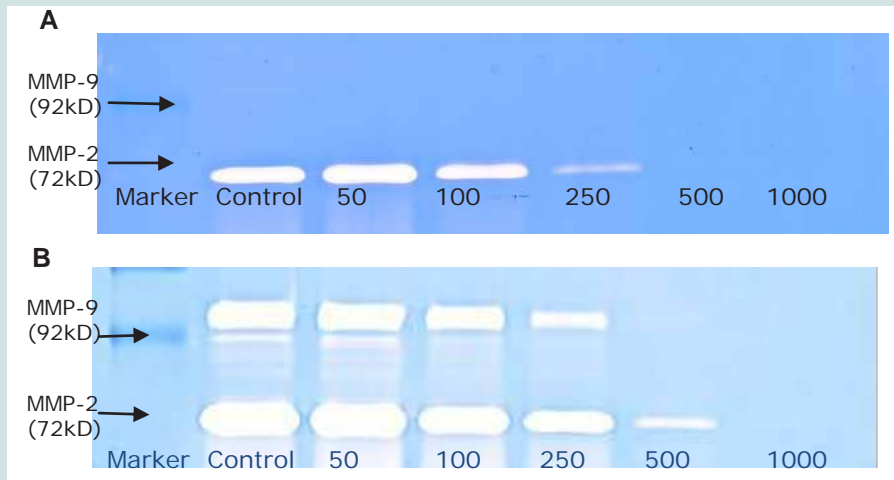


Figure 2: A) Effect of Nutrient Mixture on constitutive MMP-2 and MMP-9 expression in human cervical cancer cells (HeLa). B) Effect of Nutrient Mixture on MMP-2 and MMP-9 expression in human cervical cancer cells (HeLa) after PMA stimulation.

affecting males 55 years and older. Prostate cancer is more common in African-American males than white males and occurs more frequently in developed countries. Developed countries account for 75%; the highest rates are found in Europe, North America, and Australia. Current diagnostic methods, e.g. PSA testing and digital rectal exam have helped in early detection of prostate cancer. Standard treatment consists of surgery (prostatectomy), hormonal therapy, and radiotherapy.

We investigated the effect of NM *in vitro* on two androgen-insensitive human prostate cancer cell lines, PC-3 and DU-145, and one androgen-sensitive cell line, LNCaP. Results demonstrate that Du-145 cell line does not secrete MMP-2 or MMP-9. PMA stimulated the secretion of MMP-9 in DU-145. MMP-9 secretion is inhibited by NM in a dose-dependent manner, with a complete inhibition at 500 µg/mL NM (Table 3). The androgen insensitive cell line PC-3 secretes both MMP-2 and MMP-9 with and without PMA stimulation. In both cases, NM can inhibit MMP-2 and MMP-9 secretion in a dose-dependent manner with complete inhibition occurring at 100-500 µg/mL NM (Table 3; Figures 3A and 3B). The androgen-sensitive cell line LNCaP secreted neither MMP-2 nor MMP-9 in the absence or presence of PMA.

Testicular cancer: It is one of the most frequently occurring cancers in young men. The incidence in whites is greater than in African-American males. Peak frequency is in early adulthood, between the ages of 20-35, and uncommon after the age of 40. Risk factors for testicular cancer include undescended testes, Klinefelter syndrome, and HIV-positive patients. Chemotherapy has been shown to eradicate testicular cancer in a number of cases and life expectancy is long [1-4].

We investigated the effect of NM on the secretion of MMPs in human testicular cancer cell line NTER-2. Results show that this cell line constitutively secretes only MMP-2, and MMP-9 secretion can be achieved under PMA stimulation. Whether with or without PMA stimulation, both MMP-2 and MMP-9 secretion decreased by the NM in a dose-dependent manner, with a complete inhibition at 100-500 µg/mL NM concentration (Table 3).

Male breast cancer: Breast cancer is less common in men and constitutes less than 1% of all breast cancers. The incidence of male breast cancer is increasing and infiltrating ductal carcinoma is the most common type of male breast cancer. However due to less breast tissue in males, the breast cancer is already spread to distant organs at the time of diagnosis, reducing the chances of complete recovery.

We investigated the effect of NM on MMP secretion in male breast cancer cell line colo-824. This cell line showed only one band corresponding to MMP-9, which was enhanced by PMA stimulation.

MMP-9 secretion was inhibited by NM in a dose dependent fashion showing a faint band at 500 µg/ml and virtually 100% inhibition at 1000 µg/ml (data not shown).

Lung cancer and mesothelioma

Lung cancer: Lung cancer is the most common form of cancer diagnosed in the US, and has one of the lowest survival rates due to high metastatic potential and poor results with standard treatments [1-4]. Lung cancer rates are increasing throughout the world, including Asia, South America, and Africa. The risk factors include cigarette smoking, occupational exposure to asbestos, radon, chromium, nickel, and diesel exhaust.

We investigated the effect of the NM on the human lung cancer cell line A-549. Constitutive MMP-2 and MMP-9 secretion, as well as PMA-induced MMP-9 secretion, was inhibited with NM treatment in a dose-dependent manner with complete MMP suppression occurring at a NM concentration of 500 µg/ml (Table 4; Figures 4A and 4B).

Malignant mesothelioma: Malignant mesothelioma is a highly aggressive tumor that arises from mesothelial lining of pleural cavities. Due to its extensive metastatic potential at the time of diagnosis, the median survival for patients with malignant mesothelioma is estimated to be 4 to 18 months. Epidemiological evidence suggests that, due to heavy use of asbestos until 1980, the number of men in Western Europe dying due to malignant mesothelioma each year will increase from 5,000 in 1998 to 9,000 in 2018. Single and multiple

Table 3: Effect of nutrient mixture on MMP-2 and MMP-9 expression in male cancers.

		MMP-2						MMP-9					
1. Prostate Cancers		0 µg/mL	10 µg/mL	50 µg/mL	100 µg/mL	500 µg/mL	1000 µg/mL	0 µg/mL	10 µg/mL	50 µg/mL	100 µg/mL	500 µg/mL	1000 µg/mL
i) Du-145	No PMA	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	With PMA	0%	0%	0%	0%	0%	0%	100%	97%	42%	10%	0%	0%
ii) PC-3	No PMA	100%	84%	20%	8%	0%	0%	100%	81%	35%	24%	0%	0%
	With PMA	100%	78%	45%	0%	0%	0%	100%	105%	125%	39%	0%	0%
iii) LNCAP	No PMA	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	With PMA	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		MMP-2						MMP-9					
2. Testicular Cancer		0 µg/mL	10 µg/mL	50 µg/mL	100 µg/mL	500 µg/mL	1000 µg/mL	0 µg/mL	10 µg/mL	50 µg/mL	100 µg/mL	500 µg/mL	1000 µg/mL
NTER-2	No PMA	100%	110%	42%	23%	0%	0%	0%	0%	0%	0%	0%	0%
	With PMA	100%	77%	28%	10%	0%	0%	100%	63%	28%	0%	0%	0%

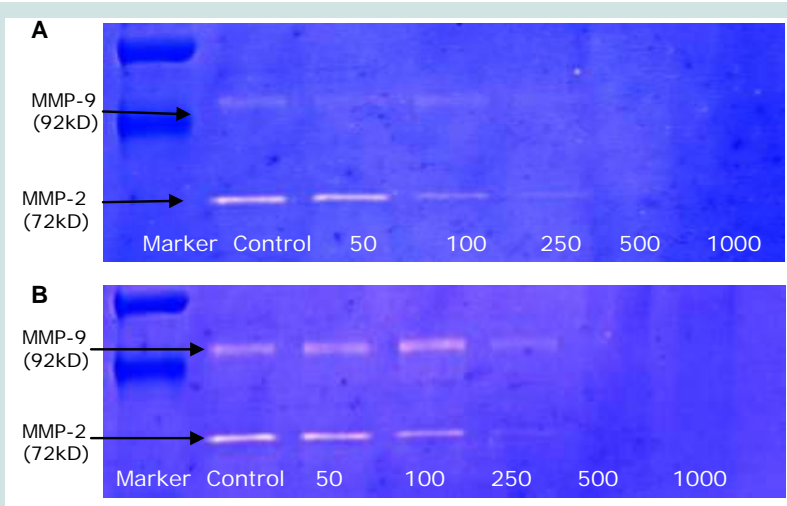


Figure 3: A) Effect of Nutrient Mixture on constitutive MMP-2 and MMP-9 expression in human prostate cancer cells (PC-3). B) Effect of Nutrient Mixture on MMP-2 and MMP-9 expression in human prostate cancer cells (PC-3) after PMA stimulation.

treatment modalities, such as surgery, chemotherapy, and radiation have failed to substantially alter this statistic [1-4].

We investigated the effect of NM on human malignant mesothelioma cell line MSTO-211H. NM significantly inhibited MMP-2 and MMP-9 secretion with and without PMA treatment in a dose-dependent manner. Given the lack of viable treatment options for this highly aggressive cancer, it is particularly significant that complete suppression of MMP secretion in MSTO-211H was achieved at a NM concentration of 500 µg/ml (Table 4).

Gastrointestinal cancers (colon, hepatocellular, pancreatic)

Colon cancer: Colon cancer, the second leading cause of cancer death in the United States, causes 55,000 deaths per year. More than 90% of colon cancer occurs in individuals over 50 years old. Risk factors include obesity, smoking, and consumption of alcohol, red meat, fatty diets, diets low in fiber, and diets low in calcium. Surgery is effective in only about 50% of the cases and the five-year survival rates are between 50-60%.

We investigated the effect of NM on colon cancer cell line

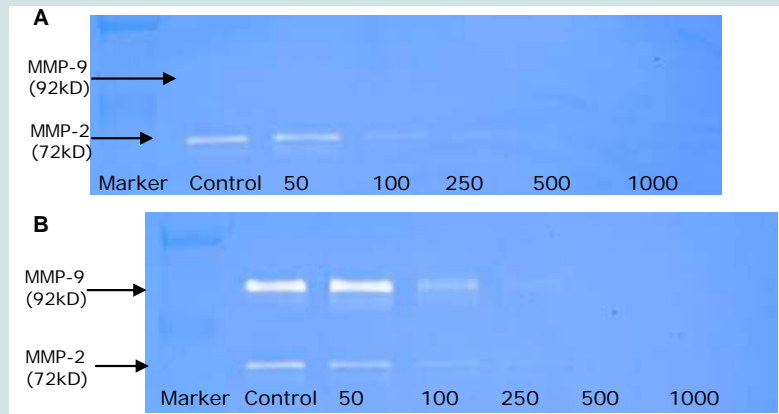


Figure 4: A) Effect of Nutrient Mixture on constitutive MMP-2 and MMP-9 expression in human lung adenocarcinoma (A-549). B) Effect of Nutrient Mixture on MMP-2 and MMP-9 expression in human lung adenocarcinoma (A-549) after PMA stimulation.

Table 4: Effect of nutrient mixture on MMP-2 and MMP-9 expression in lung cancer and mesothelioma.

		MMP-2						MMP-9						
		0 µg/mL	10 µg/mL	50 µg/mL	100 µg/mL	500 µg/mL	1000 µg/mL	0 µg/mL	10 µg/mL	50 µg/mL	100 µg/mL	500 µg/mL	1000 µg/mL	
1. Lung Adeno-carcinoma (A-549)	No PMA	100%	87%	20%	6%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	With PMA	100%	60%	12%	2%	0%	0%	100%	115%	14%	2%	0%	0%	0%
2. Mesothelioma (MSTO-211H)	No PMA	100%	111%	124%	99%	2%	0%	100%	73%	57%	34%	0%	0%	
	With PMA	100%	122%	94%	51%	0%	0%	100%	109%	100%	41%	0%	0%	

HCT-116. HCT-116 did not secrete MMP-2 either constitutively or with PMA stimulation; MMP-9 was secreted constitutively. The NM inhibited MMP-9 secretion in a dose-dependent manner with complete suppression occurring at 100 µg/ml NM concentration (Table 5).

Liver cancer: Primary liver cancer, which originates predominantly from the parenchymal liver cells or hepatocytes, includes hepatocellular carcinoma, angiosarcoma, cholangiocarcinoma, and hepatoblastoma. Hepatocellular carcinoma (HCC) is the most predominant, accounting for 90% of liver cancers. Hepatitis B and C viruses increase the risk of primary liver cancer. Other risk factors include excessive alcohol consumption and exposure to chemicals such as aflatoxin B1, androgenic steroids, and oral contraceptives. Other types of liver cancer are less common.

We investigated the effect of NM on two human HCC cell lines, Hep-G2 and SK-Hep-1. NM inhibited secretion of both MMP-2 and MMP-9 in the presence and absence of PMA, in a dose-dependent manner. For both cancer cell lines, a complete inhibition of MMP-2 and MMP-9 secretion occurred at a NM concentration of 500-1000 µg/m (Table 5; Figures 5A and 5B).

Pancreatic cancer: Cancer of the pancreas pancreatic cancer is the thirteenth common cancer in the world. Although uncommon,

pancreatic cancer is the fourth leading cause of cancer-related deaths in both men and women and is almost always fatal. Pancreatic cancer causes approximately 28,000 deaths in the US and 50,000 deaths in Europe each year.

We investigated the effect of NM *in vitro* on human pancreatic cancer cell line MIA-PaCa-2. MIA-PaCa-2 does not secrete MMP-2 either constitutively or with PMA stimulation. However, secretion of MMP-9 was inhibited by the NM in a dose-dependent manner with its complete suppression at 1000 µg/mL (Table 5).

Head and neck cancer (laryngeal, tongue, thyroid)

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy in the US and has a yearly incidence of 40,000 to 50,000 cases [1-4]. Ninety percent of these cancers involve squamous cell histology. The most common sites are the oral cavity, pharynx, larynx, and hypopharynx. HNSCC are known for their aggressive growth and propensity to invade and metastasize. Smoking tobacco, chewing tobacco, and alcohol are major risk factors for developing HNSCC. Other risk factors include sun exposure, occupational exposure to nickel, radium, chromium, mustard gas, leather, wood dust, radiation, EBV infections, and disorders of DNA repair such as Fanconi anemia. HNSCC is one of the major causes of mortality and morbidity in Fanconi anemia (FA) patients.

Table 5: Effect of nutrient mixture on MMP-2 and MMP-9 expression in gastrointestinal cancers.

		MMP-2						MMP-9					
		0	10	50	100	500	1000	0	10	50	100	500	1000
		µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL
1. Hepatocellular Carcinoma	No PMA	100%	119%	75%	35%	2%	0%	100%	107%	67%	36%	7%	0%
	w/PMA	100%	83%	48%	27%	0%	0%	100%	81%	79%	21%	19%	0%
ii) Hep-G2	No PMA	100%	71%	71%	34%	0%	0%	0%	0%	0%	0%	0%	0%
	w/PMA	100%	83%	48%	27%	0%	0%	100%	81%	77%	76%	110%	0%
		MMP-2						MMP-9					
2. Pancreatic Cancer		0	10	50	100	500	1000	0	10	50	100	500	1000
		µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL
MIA-PaCa-2	No PMA	0%	0%	0%	0%	0%	0%	100%	34%	32%	26%	24%	0%
	w/PMA	0%	0%	0%	0%	0%	0%	100%	93%	76%	21%	3%	1%
		MMP-2						MMP-9					
3. Colon Cancer		0	10	50	100	500	1000	0	10	50	100	500	1
		µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	0
HCT-116	No PMA	0%	0%	0%	0%	0%	0%	100%	60.3%	33.58%	0%	0%	0%
	w/PMA	0%	0%	0%	0%	0%	0%	-	-	-	-	-	-

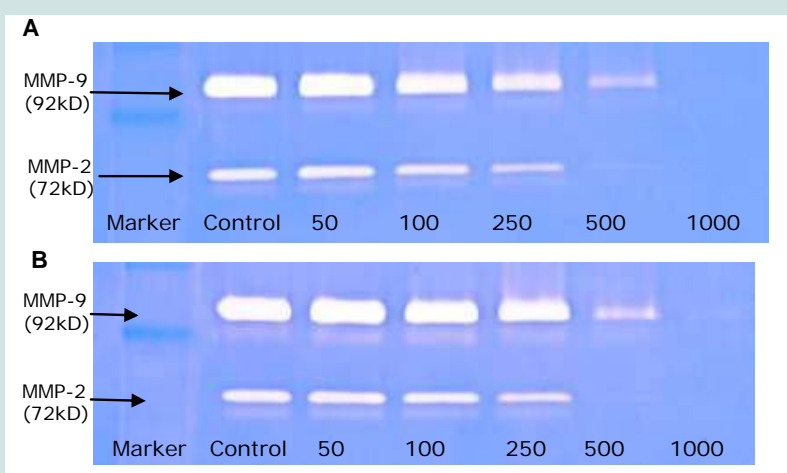


Figure 5: A) Effect of Nutrient Mixture on constitutive MMP-2 and MMP-9 expression in human hepatocellular carcinoma (SK-Hep-1), B) Effect of Nutrient Mixture on MMP-2 and MMP-9 expression in human hepatocellular carcinoma (SK-Hep-1) after PMA stimulation.

We investigated the antineoplastic effects of NM on human FA HNSCC cell line cell line OHSU-974. Zymography revealed MMP-2 and PMA-induced MMP-9 secretion. NM suppressed secretion of both MMPs in a dose-dependent manner with virtual inhibition at 500 µg/ml.

The NM was also effective in affecting MMP secretion in laryngeal squamous cell carcinoma (FaDu), tongue carcinoma (SCC-25), and thyroid carcinoma (SW-579). Both MMP-2 and MMP-9 were inhibited in a dose-dependent manner before and after PMA stimulation in FaDu (Table 6) and SCC-25 (Table 6; Figures 6A and

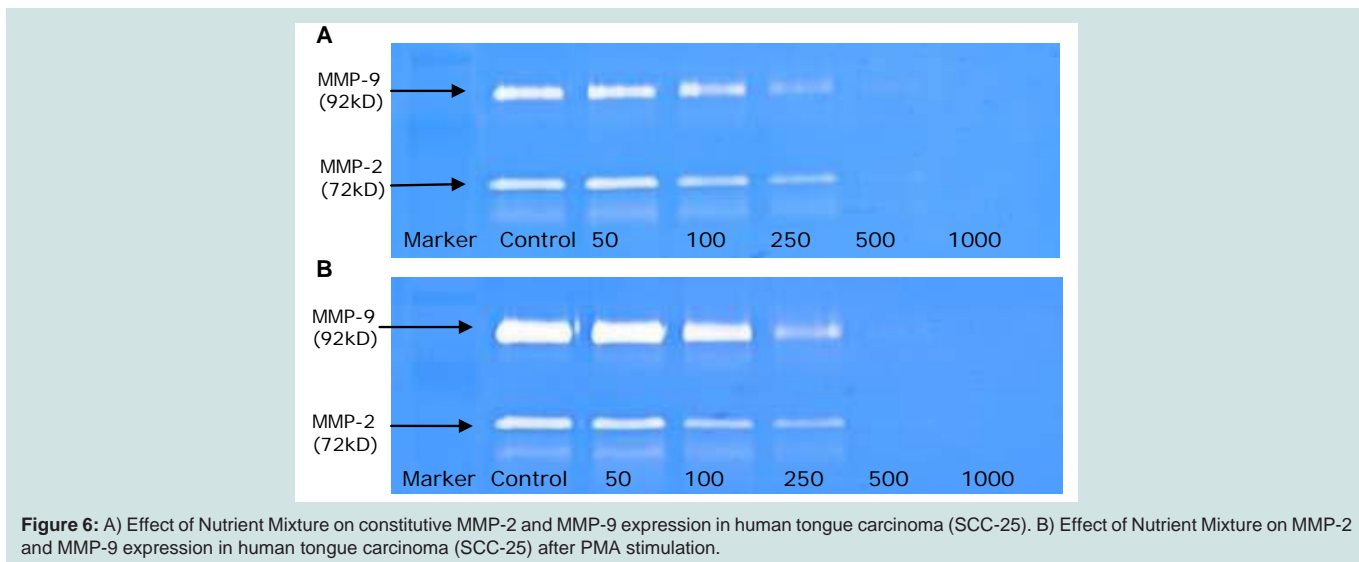
6B). The same dose-dependent inhibition was observed in SW-579 only for MMP-2 since this cell line did not secrete MMP-9 even after PMA stimulation. Complete suppression of MMP secretion in FaDu required a NM concentration of 500-1000 µg/ml and 1000 µg/m in both SCC-25 and SW-579 (Table 6).

Sarcomas

Adult sarcomas: (Liposarcoma, fibrosarcoma, chondrosarcoma, synovial sarcoma) Liposarcoma, a malignancy of fat cells, is the most common soft tissue sarcoma with an annual incidence of 2.5 cases per

Table 6: Effect of nutrient mixture on MMP-2 and MMP-9 expression in head and neck cancers.

		MMP-2						MMP-9					
		0 µg/mL	10 µg/mL	50 µg/mL	100 µg/mL	500 µg/mL	1000 µg/mL	0 µg/mL	10 µg/mL	50 µg/mL	100 µg/mL	500 µg/mL	1000 µg/mL
Laryngeal squamous carcinoma (FaDu)	No PMA	100%	83%	80%	66%	0%	0%	0%	0%	0%	0%	0%	0%
	With PMA	100%	88%	44%	13%	0%	0%	100%	82%	81%	54%	4%	0%
Tongue Carcinoma (SCC-25)	No PMA	100%	100%	54%	25%	1%	0%	100%	100%	55%	11%	1%	0%
	With PMA	100%	79%	35%	21%	2%	0%	100%	93%	53%	11%	1%	0%
Thyroid Carcinoma (SW-579)	No PMA	100%	100%	100%	73%	19%	2%	0%	0%	0%	0%	0%	0%
	With PMA	100%	109%	93%	72%	19%	1%	0%	0%	0%	0%	0%	0%



million. Fibrosarcoma, an aggressive and highly metastatic cancer of the connective tissue, primarily develops in the metaphyses of long tubular bones and affects both children and adults. Chondrosarcoma, a malignant tumor of cartilage cells mainly affecting adults between 30-60 years and accounting for more than 40% of the adult primary bone tumor cases. The poor prognosis associated with fibrosarcoma and chondrosarcoma can be attributed to both, the aggressive metastatic spread characteristic of these cancers and lack of efficacy of current treatments [1-4].

We investigated the effect of NM *in vitro* on chondrosarcoma cell line SW-1353, fibrosarcoma HT-1080, liposarcoma SW-872, and synovial sarcoma SW-982. The secretion of MMP-2 and MMP-9, both constitutive and PMA-stimulated, was inhibited in a dose-dependent manner by NM with complete suppression requiring a NM concentration of 500-1000 µg/ml (Table 7). The complete suppression of MMP secretion in the particularly aggressive fibrosarcoma line HT-1080 is a particularly encouraging result (Table 7, Figures 7A and 7B).

Pediatric sarcomas (Osteosarcoma, rhabdomyosarcoma): Malignancies of the soft tissues (6%) and bone (5%) account for more than 10% of diagnosed cancers in children, adolescents, and young adults. Benign musculoskeletal neoplasms are more common than malignant soft tissue tumors [1-4]. Rhabdomyosarcoma, the most common soft tissue sarcoma, is found predominantly in males. It is mesenchymal in origin, and affects primarily infants and children up to five years of age. On the other hand, osteosarcoma accounts for about 60% of malignant bone tumors between the ages of 10-20 years of age. The remaining bone malignancies in children and adolescents are Ewing sarcoma, a soft tissue cancer that most often occurs around the leg or arm joint and has a 50% rate of metastasis.

We investigated the effect of NM on osteosarcoma cell lines MNNG-HOS, SK-ES-1, and U-2OS, and rhabdomyosarcoma cell line RD. All the four cell lines secreted MMP-2 and MMP-9 constitutively and under PMA stimulation. The NM inhibited MMP-2 secretion in a dose-dependent manner with its complete suppression at 500 µg/

Table 7: Effect of nutrient mixture on MMP-2 and MMP-9 expression in adult sarcomas.

A. Adult Sarcomas			MMP-2						MMP-9					
			0	10	50	100	500	1000	0	10	50	100	500	1000
			µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL
1.	Chondrosarcoma (SW-1353)	No PMA	100%	100%	93%	57%	3%	0%	100%	100%	25%	19%	0%	0%
		With PMA	100%	141%	119%	26%	0%	0%	100%	108%	116%	83%	1%	0%
2.	Fibrosarcoma (HT-1080)	No PMA	100%	99%	99%	66%	36%	17%	100%	81%	35%	24%	0%	0%
		With PMA	100%	103%	49%	63%	2%	0%	100%	104%	98%	106%	45%	2%
3.	Liposarcoma (SW-872)	No PMA	100%	100%	88%	45%	1%	0%	100%	100%	64%	12%	0%	0%
		With PMA	100%	130%	75%	14%	0%	0%	100%	103%	83%	59%	3%	0%
4.	Synovial Sarcoma (SW-982)	No PMA	100%	97%	49%	7%	0%	0%	100%	0%	0%	0%	0%	0%
		With PMA	100%	105%	49%	4%	0%	0%	100%	46%	3%	0%	0%	0%

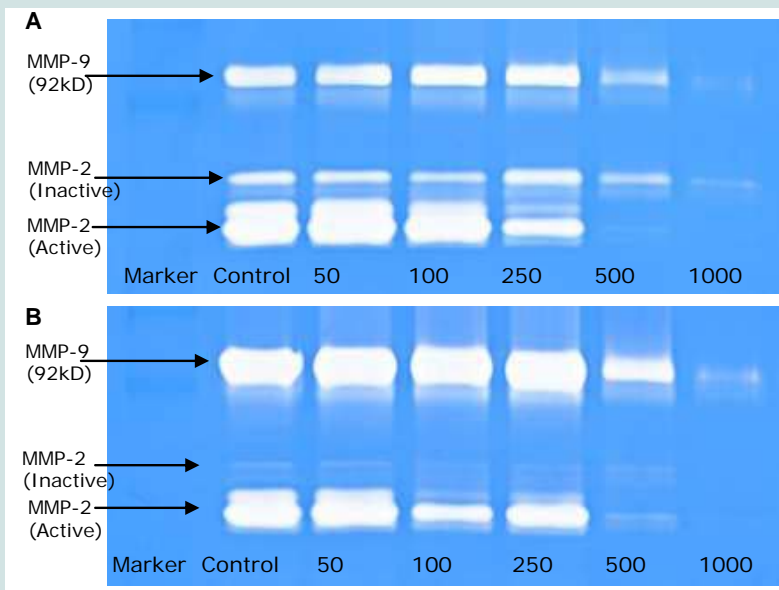


Figure 7: A) Effect of Nutrient Mixture on constitutive MMP-2 and MMP-9 expression in human fibrosarcoma (HT-1080). B) Effect of Nutrient Mixture on MMP-2 and MMP-9 expression in human fibrosarcoma (HT-1080) after PMA stimulation.

ml (Table 8). U-2OS and RD cells secreted MMP-9 constitutively. MMP-9 secretion in these cancer cell lines was much more sensitive to suppression by the NM requiring as little as 10 µg/ml of NM for complete inhibition. MNNG-HOS and SK-ES-1 cell lines secreted MMP-9 only after PMA stimulation, which was inhibited by NM in a dose-dependent manner with a complete inhibition at 500 µg/ml NM (Table 8).

Renal and urinary cancers (kidney, bladder)

Renal cancer: The American Cancer Society estimates approximately 65,150 new cases of kidney cancer in 2013. Upon diagnosis, renal cancer cell carcinoma (RCC) is localized to the

kidney in 50% of the cases; in another 25%, the cancer will spread to nearby tissue and in the remaining 25% will have metastasized to distal sites. Associated 5-year survival in these patient groups is roughly 90%, 60%, and 9%, respectively. In the United States, the incidence of renal cell carcinoma is higher among African American population. Smoking contributes to one third of all cases of RCC. Other contributing factors are high consumption of fried fatty meat, obesity (particularly in women), and exposure to asbestos and petroleum products. Chemotherapy and radiation are rarely effective for people with renal cancer.

Bladder cancer: Bladder cancer is the 11th most common cancer in the world, with over 50% of the cases found in developed countries

Table 8: Effect of nutrient mixture on MMP-2 and MMP-9 expression in pediatric sarcomas.

B. Pediatric Sarcomas		MMP-2						MMP-9					
		0 µg/mL	10 µg/mL	50 µg/mL	100 µg/mL	500 µg/mL	1000 µg/mL	0 µg/mL	10 µg/mL	50 µg/mL	100 µg/mL	500 µg/mL	1000 µg/mL
Osteosarcoma (MNNG-HOS)	No PMA	100%	116%	6%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	With PMA	100%	113%	3%	0%	0%	0%	100%	174%	7%	0%	0%	0%
Osteosarcoma (SK-ES-1)	No PMA	100%	68%	7%	2%	0%	0%	0%	0%	0%	0%	0%	0%
	With PMA	100%	70%	8%	4%	0%	0%	100%	115%	0%	0%	0%	0%
Osteosarcoma (U-2OS)	No PMA	100%	92%	48%	4%	0%	0%	100%	81%	3%	0%	0%	0%
	With PMA	100%	90%	21%	8%	0%	0%	100%	108%	86%	79%	1%	0%
Rhabdomyosarcoma (RD)	No PMA	100%	104%	73%	60%	0%	0%	100%	0%	0%	0%	0%	0%
	With PMA	100%	130%	83%	55%	0%	0%	100%	97%	81%	64%	2%	1%

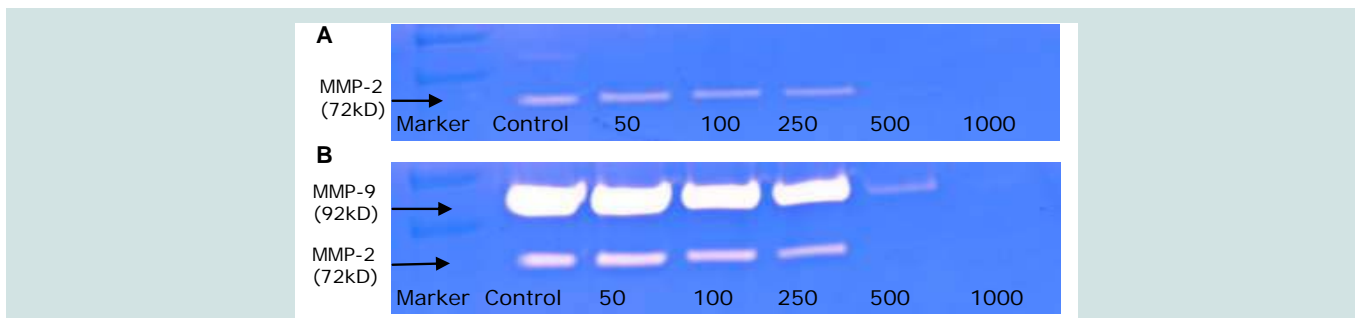


Figure 8: A) Effect of Nutrient Mixture on constitutive MMP-2 expression in human embryonic rhabdomyosarcoma (RD). B) Effect of Nutrient Mixture on MMP-2 and MMP-9 expression in human embryonic rhabdomyosarcoma (RD) after PMA stimulation.

[1-4,56]. Smokers have twice the risk of developing bladder cancer than non-smokers. In addition, people exposed to dye, paint, rubber, leather, and hair chemicals are at a greater risk.

We investigated the effect of NM on MMP-2 and MMP-9 secretion in renal cell adenocarcinoma cell line RCC-786-0 and bladder cancer cell line T-24. RCC-786-0 secreted both these enzymes constitutively (Table 9, Figures 9A and 9B). T-24 cells secreted MMP-2 and MMP-9 only after PMA-induction. The NM inhibited secretion of both MMP-2 and MMP-9 either before or after PMA stimulation with complete suppression occurring at a concentration of 100-500 µg/ml (Table 9).

Cancers of the nervous system (glioblastoma, neuroblastoma, retinoblastoma)

Glioblastoma: The most common and most aggressive malignant primary brain tumor is glioblastoma multiforme. Glioblastoma multiforme is a grade IV astrocytoma, a tumor arising from glial cells surrounding the neurons. Unfortunately, prognosis is very poor and median survival time is approximately 14 months after diagnosis with glioblastoma multiforme [1-4].

Neuroblastoma: It is the most common extra cranial solid cancer in childhood and the most common cancer in infancy, with an annual incidence of about 650 new cases per year in the US. Neuroblastoma is a neuroendocrine tumor arising most frequently occurring in adrenal glands although it can develop in nerve tissues of the neck, chest, abdomen, or pelvis. Prognosis is varied. In very young children,

spontaneous remission can occur; in other cases, metastasis to bones (including bone marrow), liver, lymph nodes, and skin is seen. Unfortunately, successful treatment often leaves children at risk for getting a second different kind of cancer in the future.

Retinoblastoma: Retinoblastoma is a cancer of the retina that can occur in hereditary or sporadic forms. Retinoblastoma usually affects children under the age of six, with most diagnoses in children around 1-2 years of age. Prognosis is excellent if metastasis has not occurred. Cure rates of around 95-98% are seen in the developed world.

We studied the effects of NM on MMP2 and MMP-9 secretion by glioblastoma cell lines A-172, LN-18, and T-98G, neuroblastoma cell line SK-N-MC, and retinoblastoma cell line Y-79. In the glioblastoma and neuroblastoma cell lines, MMP-2 was secreted constitutively while MMP-9 required PMA treatment. After exposure to the NM, both in the presence and absence of PMA, MMP-2 and MMP-9 secretion was inhibited in a dose-dependent manner in all four of these cell lines with complete suppression requiring 50-100 µg/ml NM concentration (Table 10; Figures 10A and 10B). Retinoblastoma cells, Y-79 secreted only MMP-2, with no MMP-9 induction after PMA treatment. The secretion of this enzyme was inhibited by NM in a dose-dependent manner with complete suppression at 1000 µg/ml of NM (Table 10).

Melanoma

Melanoma is a skin tumor that originates from the melanocytes and ranks as the seventh leading type of cancer in the US. Its incidence

Table 9: Effect of nutrient mixture on MMP-2 and MMP-9 expression in nephro-urologic cancers.

		MMP-2						MMP-9					
		0	10	50	100	500	1000	0	10	50	100	500	1000
		µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL
Bladder Carcinoma (T-24)	No PMA	100%	175%	77%	21%	0%	0%	0%	0%	0%	0%	0%	0%
	With PMA	100%	42%	23%	0%	0%	0%	100%	103%	28%	0%	0%	0%
Renal Adenocarcinoma (RCC-786-0)	No PMA	100%	93%	37%	37%	0%	0%	100%	53%	27%	27%	0%	0%
	With PMA	100%	122%	56%	4%	0%	0%	100%	121%	88%	14%	0%	0%

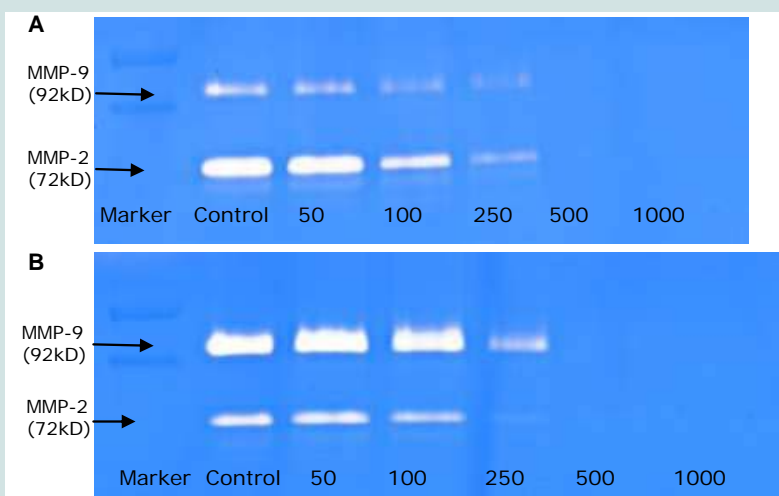


Figure 9: A) Effect of Nutrient Mixture on constitutive MMP-2 expression in human renal adenocarcinoma (RCC-786-0). B) Effect of Nutrient Mixture on MMP-2 and MMP-9 expression in human renal adenocarcinoma (RCC-786-0) after PMA stimulation.

Table 10: Effect of nutrient mixture on MMP-2 and MMP-9 nervous system cancers.

		MMP-2						MMP-9					
		0	10	50	100	500	1000	0	10	50	100	500	1000
		µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL
Glioblastoma i) A172	No PMA	100%	100%	94%	67%	3%	0%	0%	0%	0%	0%	0%	0%
	With PMA	100%	62%	34%	38%	0%	0%	100%	100%	100%	100%	0%	0%
ii) LN-18	No PMA	100%	178%	66%	13%	0%	0%	0%	0%	0%	0%	0%	0%
	With PMA	100%	140%	44%	0%	0%	0%	100%	165%	0%	0%	0%	0%
iii) T-98G	No PMA	100%	89%	52%	22%	6%	2%	0%	0%	0%	0%	0%	0%
	With PMA	100%	118%	70%	16%	4%	0%	100%	63%	4%	0%	0%	0%
MMP-2							MMP-9						

		0	10	50	100	500	1000	0	10	50	100	500	1000
		µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL
Neuroblastoma (SK-N-MC)	No PMA	100%	136%	50%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	With PMA	100%	142%	27%	0%	0%	0%	100%	115%	36%	0%	0%	0%
		MMP-2						MMP-9					
		0	10	50	100	500	1000	0	10	50	100	500	1000
		µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL
Retinoblastoma	No PMA	100%	122%	100%	104%	12%	0%	0%	0%	0%	0%	0%	0%
	With PMA	100%	126%	114%	92%	12%	0%	0%	0%	0%	0%	0%	0%

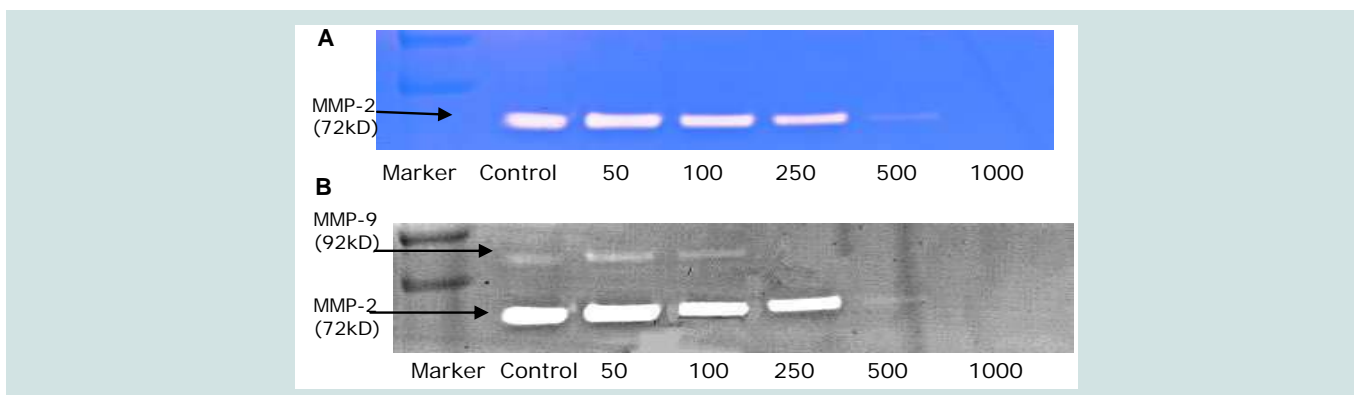


Figure 10: A) Effect of Nutrient Mixture on constitutive MMP-2 expression in human glioblastoma cells (A-172). B) Effect of Nutrient Mixture on MMP-2 and MMP-9 expression in human glioblastoma cells (A-172) after PMA stimulation.

is ten times higher in Caucasians than in African-Americans. Sun exposure is the principle cause of melanoma. Individuals with fair skin, blue eyes, and blonde or red hair are at highest risk for melanoma. Prognosis varies depending on the stage of the malignancy at the time of diagnosis. For small lesions, surgical resection can be an effective cure. At the other extreme, metastatic malignant melanoma of stage III can have a 5-year survival rate as low as 27% [1-4].

We investigated the effect of NM on melanoma cell line A-2058. *In vitro*, A-2058 secreted only MMP-2 and after PMA stimulation –the MMP-9 only. Secretion of both enzymes without and after PMA treatment was inhibited by the NM in a dose-dependent manner. Complete suppression of MMP-2 required 1000 µg/ml NM concentration, while MMP-9 suppression was observed at 500 µg/ml of NM (Table 11; Figures 11A and 11B).

Hematologic cancers

Leukemia is a malignancy of bone marrows cells in which clones of immature blood cells multiply at the expense of normal blood cells. As normal blood cells are depleted, anemia, infection, hemorrhage, and eventually death can occur. The peak incidence of Acute Promyelocytic Leukemia (APL) occurs at age of 40 years old whereas T-cell Acute Lymphoblastic Leukemia (T-ALL) between ages 2-5 years. Currently, APL is successfully treated with a combination of vitamin A and the antibiotic anthracycline to achieve a 90% remission rate. T-ALL is currently treated with systemic and CNS chemotherapy, followed by consolidation therapy, totaling 2 years

of treatment. Complete remission is seen in approximately 98% of children, and approximately 85% of adults.

Lymphoma is a malignant neoplasm of lymphocytes. Lymphomas can be of the Hodgkin type (defined by the presence of Reed-Sternberg cells) or non-Hodgkin type. Burkitt Lymphoma (a non-Hodgkin lymphoma of B-cells) is most common in equatorial Africa affecting children between the ages of 5 and 10 and is strongly associated with endemic EBV infections [1-4].

We studied the effects of NM on acute promyelocytic leukemia cell line HL-60, acute lymphoblastic T-cell leukemia cell line Jurkat, and Burkitt’s lymphoma cell line Raji. HL-60 secreted MMP-2 constitutively, and MMP-9 only after PMA stimulation. Jurkat (Table 12) and Raji (Table 12; Figures 12A and 12B) did not secrete MMP-2 either constitutively or with PMA stimulation. Jurkat Secretion of MMP-9 was observed in Jurkat cell line after PMA-stimulation, while secretion of MMP-9 occurred constitutively and when stimulated with PMA in Raji cells. In all cell lines tested MMP-2 and MMP-9 secretion was inhibited in a dose-dependent manner after exposure to NM with a complete suppression at a NM concentration of 1000 µg/m (Table 12).

Discussion

The aggressiveness of a particular type of cancer is roughly proportional to the rate of tumorigenesis and metastasis [8-21]. The major events of cancer proliferation are cell attachment, angiogenesis to provide the tumor with nutrients, degradation of the ECM to

Table 11: Effect of nutrient mixture on MMP-2 and MMP-9 expression in melanoma.

Melanoma		MMP-2						MMP-9					
		0 µg/mL	10 µg/mL	50 µg/mL	100 µg/mL	500 µg/mL	1000 µg/mL	0 µg/mL	10 µg/mL	50 µg/mL	100 µg/mL	500 µg/mL	1000 µg/mL
A-2058	No PMA	100%	113%	92%	27%	12%	0%	0%	0%	0%	0%	0%	0%
	With PMA	100%	129%	87%	32%	1%	0%	100%	87%	5%	1%	0%	0%

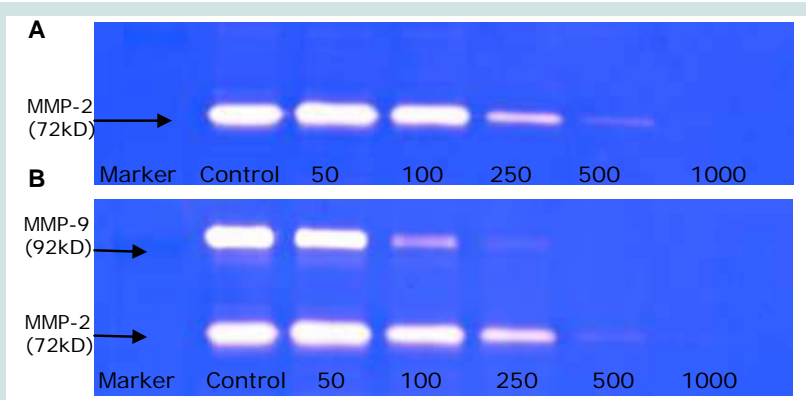


Figure 11: A) Effect of Nutrient Mixture on constitutive MMP-2 expression in human melanoma cells (A-2058). B) Effect of Nutrient Mixture on MMP-2 and MMP-9 expression in human melanoma cells (A-2058) after PMA stimulation.

Table 12: Effect of nutrient mixture on MMP-2 and MMP-9 expression in leukemias.

Leukemias		MMP-2						MMP-9					
		0 µg/mL	10 µg/mL	50 µg/mL	100 µg/mL	500 µg/mL	1000 µg/mL	0 µg/mL	10 µg/mL	50 µg/mL	100 µg/mL	500 µg/mL	1000 µg/mL
i) Leukemia Promyelocytic (HL-60)	No PMA	100%	80%	51%	60%	27%	0%	0%	0%	0%	0%	0%	0%
	With PMA	100%	96%	85%	58%	11%	0%	100%	110%	75%	56%	9%	1%
ii) Leukemia T-ALL (Jurkat)	No PMA	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	With PMA	0%	0%	0%	0%	0%	0%	100%	121%	106%	88%	0%	0%
iii) Burkitt Lymphoma (Raji)	No PMA	0%	0%	0%	0%	0%	0%	100%	87%	85%	19%	2%	0%
	With PMA	0%	0%	0%	0%	0%	0%	100%	134%	148%	146%	23%	3%

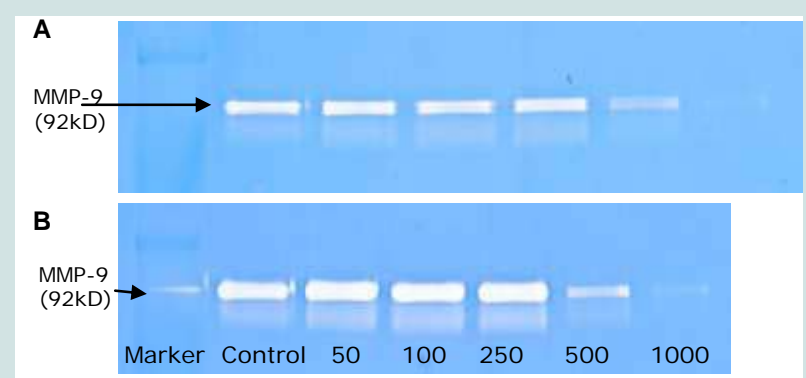


Figure 12: A) Effect of Nutrient Mixture on constitutive MMP-9 expression in Burkitt Lymphoma cell line Raji. B) Effect of Nutrient Mixture on MMP-9 expression in Burkitt Lymphoma cell line Raji after PMA stimulation.

allow tumor expansion, and migration of the tumor cells through the disrupted extracellular matrix [11]. MMPs, particularly the gelatinases MMP-2 and MMP-9, play central roles in these processes due to their ability of to degrade type IV collagen, one of the major components of the basement membranes and ECM. In fact, the aggressiveness of a cancer is often directly proportion to the overproduction of MMP-2 and MMP-9 [6-11].

Our earlier studies have shown that combining these naturally derived substances achieve a substantially more potent synergistic effect against constitutive and PMA-stimulated MMP-2 and MMP-9 secretion than any action of any of the individual nutrients [44-49]. Previous research from our institute has shown that nutrients such as ascorbic acid, lysine, and epigallocatechin gallate when tested individually have anti-proliferative and apoptotic effects on different types of leukemia cells [62-64]. While there could be other possible mechanisms, according to our previous studies when L-lysine and EGCG were separately used in in HTLV-1 positive and -negative leukemia cell, it was proven that these nutrients exert their effects through Nuclear Factor-kappaB (NF-kB) pathway [64]. Similarly, EGCG also has apoptotic action on leukemia cells and it inhibits the MMP-9 expression on both transcriptional and translational levels [64].

In this study we compared the efficacy of a nutrient mixture (NM) consisting of Vitamin C, L-lysine, L-proline, L-arginine, N-acetylcysteine, selenium, copper, manganese, and epigallocatechin gallate (EGCG) on the secretion of MMP-2 and MMP-9 *in vitro* by 42 different cancer cell lines from representative classes of organ malignancies. The select cancer cell lines studied were grouped by organ malignancies and the MMP-2 and MMP-9 secretion was determined using gelatinase zymography with MMP-2 appearing as a band at 72 kD and MMP-9 as a band at 92 kD. Some cancer cell lines secreted only MMP-2 or MMP-9 and some secreted both.

Our study has shown that NM has an inhibitory effect on MMP-2 and MMP-9 secretion in all 42 cancer lines tested. This effect was dose depended and was observed in every cancer line that secreted MMP-2 and MMP-9, either constitutively or after PMA stimulation. The effective concentrations of NM required to achieve the total inhibition of MMPs secretion varied between different cancer cell lines. In 34 of the 41 cancer cell lines a suppression of MMP-2 and MMP-9 secretion was achieved at 50 µg/ml-100 µg/ml NM concentrations in the other 7 cancer cell lines at 500 µg/ml-1000 µg/ml (Tables 2-12). In the seven cancer lines in which MMP secretion was not completely suppressed by the NM, six of them secreted MMP-9 and only one cell line secreted MMP-2. The secretion of MMP-9 in these six lines was reduced to 1%-2% of control at the maximum NM concentration employed of 1000 µg/ml (Tables 2 (MCF-7), 5 (MiaPaCa-2), 7 (HT-1080), 8 (RD), and 12 (HL-60; Burkitt, Raji)). The secretion of MMP-2 in thyroid carcinoma was inhibited by 98-99% at a 1000 µg/ml NM (Table 6). Only constitutively secreted MMP-2 in fibrosarcoma line HT-1080 was secreted at 17% of control level at a NM concentration of 1000 µg/ml (Table 7). However, the secretion of MMP-2 induced by PMA in this cell line was significantly reduced to 2% of control at a NM concentration of 500 µg/ml with its complete suppression at 1000 µg/ml.

This study demonstrates the *in vitro* efficacy of a combination of

Table 13: Cytotoxic effect of NM on various cancer cell lines.

Cell lines used	% toxicity
Prostate cancer (LNCap)	80% toxicity at 100 µg/ml
Breast cancer (MDA-MB-231)	34% toxicity at 500 µg/ml
Cervical cancer (Hela)	50% toxicity at 500 µg/ml
Uterine cancer (MES-SA)	65% toxicity at 500 µg/ml
Lung cancer (A-549)	20% toxicity at 1000 µg/ml
Liver cancer (SK-HEP-1)	33% toxicity at 500 µg/ml
Pancreatic cancer (MiaPaCa-2)	57% toxicity at 500 µg/ml
Adult sarcoma-Fibrosarcoma (HT-1080)	20% toxicity at 500 µg/ml
Pediatric sarcoma-Osteosarcoma(MNNG-HOS)	20% toxicity at 500 µg/ml
Leukemia (Jurkat cells)	72% toxicity at 500 µg/ml
Tongue cancer (SCC-25)	73% toxicity at 500 µg/ml
Retinoblastoma (Y-79)	85% toxicity at 500 µg/ml
Melanoma (A2058)	64% toxicity at 500 µg/ml

natural substances (NM) on MMP 2 and MMP-9 secretion in multiple cancer cell lines representing a variety of cancer origins and types. The previous *in vivo* studies conducted by our group demonstrated modulation of MMP-9 secretion with NM treatment. Tumor tissue from xenograft studies showed reduction of MMP-9 secretion with NM diet [48]. Our other *in vivo* and *in vitro* studies have documented comprehensive anti-cancer effects of this nutrient combination in over 40 types of cancer cells, in particular in inducing cytotoxicity (Table 13), migration, invasion, and metastasis [58] as well as in inhibiting angiogenesis [59,60] and inducing apoptosis [61].

Given the fundamental role of MMP-2 and MMP-9 in the progression of cancer, the dose-dependent suppression of MMP-2 and MMP-9 secretion demonstrates the enormous potential of this combination of natural compounds in cancer treatment. Development of synthetic MMP inhibitors has been underway for more than thirty years. Yet, none of the numerous synthetic MMP inhibitors has been proven successful beyond the phase of clinical testing due to their poor absorption, bioavailability and undesirable side effects during the administration and with long term usage [5]. Since secretion of MMPs is crucial to the invasive potential of cancer cells, the research in MMP inhibitors continues.

In contrast to toxic side effects of current cancer treatment modalities, the NM is proven to be non-toxic. It has no known side effects on vital organs such as heart, liver, and kidney, when used in the clinically relevant doses as shown in a recent *in vivo* toxicology study [50]. NM also did not increase the functional serum enzymes indicating that it is safe to use even in high doses far exceeding the normal equivalent doses of the nutrients. Moreover, the NM reduced MMP secretion *in vitro* and *in vivo* in xenograft models and reduced chemically induced tumors in our studies [64]. NM is readily absorbed, and is low-cost compared to current cancer treatments. Therefore, we believe NM offers multiple unique benefits in the fight against cancer, worldwide.

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