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Anticancer Effects of a Micronutrient Mixture on Melanoma – Modulation of Metastasis and Other Critical Parameters

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1. Introduction

Melanoma causes the most skin cancer-related deaths, due to metastasis to other areas of the body, such as lymph nodes, lungs, liver, brain or bone. Though often curable in its early stages, metastatic malignant melanoma is an extremely aggressive cancer with no current viable treatment. Thus, any successful treatment for melanoma has to target metastasis. Invasion of host tissues by cancer cells requires alteration of cancer cell adhesion, cell migration and proteolytic degradation of the extracellular matrix (ECM) (Fidler, 1990). Dr. Rath proposed that optimizing the stability and structure of the ECM and controlling its proteolytic degradation would be the most effective and universal approach to controlling cancer invasiveness and tumor growth (Rath & Pauling, 1992). Degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs) plays a critical role in the formation of tumors and metastasis and has been found to correlate with the aggressiveness of tumor growth and invasiveness of the cancer (Fidler, 1990; Duffy, 1992; Stetler-Stevenson, 2001). Since this process is involved not only in metastasis, but also in angiogenesis and tumor growth, control of proteolytic activity of ECM provides an opportunity to modulate key common aspects of malignancy.

Rath and Pauling suggested the use of nutritional components, such as vitamin C and lysine and lysine analogues to target plasmin-mediated connective tissue degradation as a universal approach to controlling common pathomechanisms of cancer (Rath & Pauling, 1992). Lysine interferes with the activation of plasminogen into plasmin by tissue plasminogen activator (tPA) by binding to plasminogen active sites, thereby affecting the plasmin-induced MMP activation cascade (Rath & Pauling, 1992). Our subsequent studies confirmed this approach and resulted in identifying a novel formulation composed of lysine, ascorbic acid, proline and green tea extract and other micronutrients (NM) which has shown significant anticancer activity against a large number (~40) of cancer cell lines, blocking cancer growth, tissue invasion and MMP expression both *in vitro* and *in vivo* (Roomi et al., 2010). Furthermore, NM demonstrated significant antiangiogenic activity utilizing the chorioallantoic membrane (CAM) assay in chick embryos and bFGF-induced vessel growth in C57BL/6J female mice in the mouse Matrigel plug assay (Roomi et al, 2005). In addition, *in vitro*, NM decreased the expression of pro-angiogenic factors of VEGF, angiopoietin-2, bFGF, PDGD and TDGβ-1 by U2OS cells (Roomi et al., 2005).

2. Selecting active compounds for the micronutrient mixture

Individual components of the NM were chosen for their potent activity on critical physiological targets in cancer progression and metastasis, such as optimization of collagen structure and stability, inhibition of MMPs, cellular migration and invasion, and induction of apoptosis . The anticancer effects of these individual constituents of the NM have been reported in both clinical and experimental studies. Optimal ECM formation and structure requires adequate supplies of ascorbic acid and the amino acids lysine and proline, since these nutrients enable proper synthesis and hydroxylation of collagen fibers. Manganese and copper are also essential for collagen formation. Lysine also acts as a natural inhibitor of plasmin-induced proteolysis to contribute to ECM stability (Rath & Pauling, 1992; Sun et al., 2002). Green tea extract has been shown to control cancer cell growth, metastasis, angiogenesis, and other aspects of cancer progression (Kemberling et al., 2003; Sato & Matsushima, 2003; Valcic et al., 1996; Muhktar & Ahmed, 2000; Yang et al., 1998; Taniguchi et al, 1992; Hara, 2001). N-acetyl cysteine and selenium have been reported to inhibit tumor cell invasion and expression of MMP-9, as well as migration of endothelial cells through ECM (Kawakami et al., 2001; Morini et al., 1999; Yoon et al., 2001). Ascorbic acid has been reported to exert cytotoxic and antimetastatic actions on malignant cell lines (Maramag et al., 1997; Koh et al., 1998; Chen et al., 2005; Kurbacher et al, 1996); in addition, low levels of ascorbic acid have been reported in cancer patients (Nunez et al, 1995; Anthony & Schorah, 1982). 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 (Cooke & Dzau, 1997). Combining these micronutrients expands metabolic targets maximizing the biological impact with lower doses of components by nutrient synergy. Furthermore, effective lower doses of components using nutrient synergy also assure overcoming absorption barriers characteristic for high vitamin doses.

3. Nutrient synergy controls metastasis

Treatment failure of patients with melanoma is mainly due to tumor metastasis to such sites in the body as lymph nodes, lungs, liver, brain or bones. Therefore, we investigated the effectiveness of the micronutrient mixture in controlling metastasis utilizing melanoma B16FO cells *in vivo*.

3.1 Pulmonary metastasis

We investigated pulmonary metastasis in 6-7 week-old female C57BL/6 mice, which were divided into 6 groups of 6 mice each (Roomi et al, 2006a). B16FO melanoma cells (5 X 10^4/mouse), were injected via tail vein into mice in Groups 1-4 and group 5 animals were injected in the tail vein with the same number of B16FO melanoma cells pretreated with NM (500 µg/mL for 18 hours). Group 6 (untreated control) was injected with the vehicle saline. Subsequently the mice were placed on the following dietary regimens and administration routes for NM: Groups 1 and 5 were fed Purina mouse chow, the control diet; group 2 mice were given NM intraperitoneally (IP) (4 mg/mouse, 3 x/week x 2 weeks) and fed the control diet; group 4 was give NM intravenously (IV) (4 mg/mouse, 3 x/week x 2 weeks and fed the control diet. Group 6 mice were fed the control diet. Two weeks later the mice were sacrificed, and their lungs were excised, weighed, and processed for histopathologic examination. The weights of the lungs were obtained and the number of metastases and number of cells in each metastatic lesion were counted.

Lungs isolated from mice injected with 5 X 10^4 B16FO cells and fed the NM 0.5% diet (group 2) demonstrated a significant reduction in metastatic colonies (by 63%, $p<0.0001$) compared to the lungs of mice fed the control diet (group 1), as shown in Figure 1. Pulmonary colonization was inhibited by 86% ($p<0.0001$) in mice receiving NM by IP and IV injections (groups 3 and 4). The lungs from mice injected with viable melanoma cells pretreated with NM (group 5) were free from any metastasis. Thus, exposing melanoma cells to NM inhibited their ability to metastasize without the need for diet supplementation, suggesting profound changes on a cellular level. Our *in vitro* studies on B16FO cells suggest that pretreatment with 500 µg/mL NM prior to injection of the cells affected cell viability of melanoma by inhibiting cell proliferation (by 44%) and inducing profound apoptosis (over 90% of cells in apoptosis). Those in vitro results are discussed in sections 5.2 and 5.3.

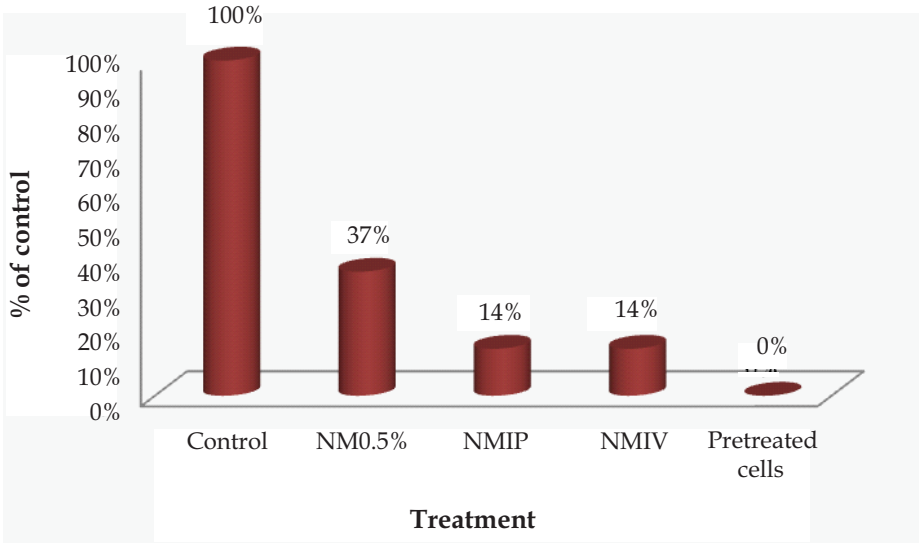


Fig. 1. Effect of NM on pulmonary colonization of B16FO cells ($5x10^4$) injected into C57BL/6 mice. Legend: NM 0.5% (NM 0.5% dietary supplementation); NM IP and NM IV (NM 4 mg/mouse, 3 x/week x 2 weeks, either IP or IV, as indicated and fed the control diet); pretreated cells (injected B16FO melanoma cells pretreated with NM 500 µg/mL for 18 hours)

3.2 Hepatic metastasis

In order to obtain a more clinically relevant model, we employed an orthotropic model of evaluating the effects of NM on hepatic metastasis. Orthotopic models have been suggested as the growth environment influences cancer behavior and thus animal models selected should recreate disease at the specific organs of interest.

In this study, athymic nude mice, 10-12 weeks old, received 10^6 B16FO melanoma cells by injection into the spleen and divided into two treatment groups (Roomi et al., 2009). The control group of mice received Purina mouse chow and the NM group received the regular diet supplemented with NM 0.5%. After two weeks, animals were sacrificed and spleens, livers, kidneys and lungs were excised from all animals, examined, weighed and processed for histology.

Mice in the control group developed large black spleens and livers indicating growth in the spleen and metastasis to the liver. However, the mice supplemented with NM not only showed less tumor growth in the spleen as the control mice, but also drastically reduced metastasis to the liver (Figures 2A-B). Intrasplenic tumor growth (mean spleen weight) was reduced significantly *(*by 64%, p=0.001) in the NM-supplemented group compared to the control group. Hepatic metastasis in NM-supplemented mice was reduced by 55% (p=0.006) compared to the control group, based on mean liver weights of the groups. In all groups, no metastasis to the kidneys and lungs was evident, Multiple, nodular, metastatic lesions replaced most of the examined liver sections of control mice. In contrast, only two to five small metastases were noted in the liver sections of NM-supplemented mice.

Fig. 2. Representative livers (A) and spleens (B) from the Control and NM-supplemented athymic mice that received intrasplenic injections of 10^6 B16FO melanoma cells

We also studied the effect of NM dietary treatment on the survival time of C57BL/6 mice after receiving an injection of 10^6 B16FO cells IP (Roomi et al., 2008). Group 1 (n=6) received no B16FO cells and regular Purina mouse chow, group 2 (n=6), 10^6 B16FO cells IP and regular Purina mouse chow, group 3, 10^6 B16FO cells IP and 0.5% NM-supplemented Purina mouse chow, and group 4, 10^6 B16FO cells IP, regular Purina mouse chow and 2 mg NM injected 3 x/week. The number of animals surviving was counted daily. The NM dietary treatment group (3) demonstrated increased mean survival time (16.7 days) over the Control diet group (2) mice with a mean survival time of 15.2 days. The longest survival time (22 days) for groups was reached by a mouse in Group 3. Group 4 mice had a mean survival time of 15.2 days.

4. Micronutrient synergy modulates invasive parameters of melanoma

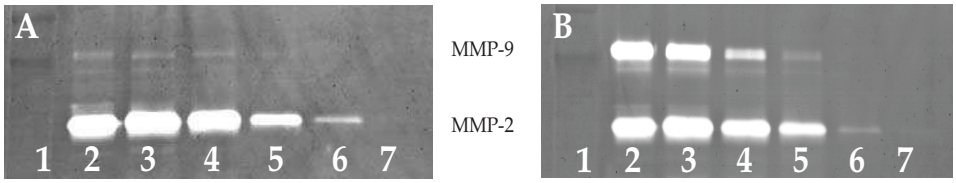
Since proteases, especially MMP-2 and MMP-9, play key roles in tumor cell invasion and metastasis, we also investigated the effects of NM *in vitro* on melanoma A2058 and B16FO MMP-2 and MMP-9 secretion (by gelatinase zymography), migration by scratch test and invasion through Matrigel. Interestingly, B16FO melanoma cells neither expressed any MMPs nor exhibit invasion through Matrigel.

4.1 MMP-2 and MMP-9 secretion

Human melanoma cells A2058 grown to confluence were treated with NM dissolved in media and tested at 0, 10, 100, 500, and 1000 μg/mL in triplicate at each dose. Parallel sets of

cultures were treated with phorbol 12-myristate 13-acetate (PMA) 100 ng/mL. Conditioned media were collected, centrifuged at 3000 rpm and the supernatant was collected and used for gelatinase zymography. Gelatinase zymography was performed in 10% Novex Pre-Cast SDS Polyacrylamide Gel (Invitrogen Corporation) 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 minutes at room temperature to remove SDS. The gels were then incubated at 37^0C overnight in substrate buffer containing 50mM Tris-HCl and 10mM $CaCl_2$ at pH 8.0 and stained with 0.5% Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid for 30 minutes and destained. Upon renaturation of the enzyme, the gelatinases digested the gelatin in the gel, producing 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 CanoScan 9950F scanner at 300 dpi.

Zymography detected MMP-2 in untreated melanoma A2058 cells and induction of MMP-9 by PMA (100 ng/mL). MMP-2 and MMP-9 expression was inhibited by NM in a dose-dependent fashion with virtual complete blockage of MMP-9 at 100 µg/ml and MMP-2 at 500 µg/mL, as shown in figures 3A-D (Roomi et al., 2010).



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

1-Marker, 2-Control, 3-7: 100ng/mL PMA
and 10, 50, 100, 500, 1000 µg/mL NM

Fig. 3. Effect of NM on MMP-2 and -9 expression by normal (A) and PMA 100 ng/mL-treated melanoma A2058 cells: gelatinase zymography (B)

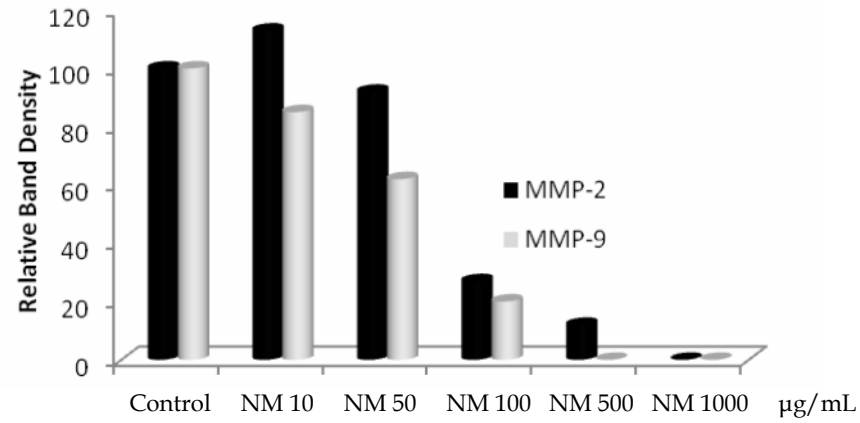


Fig. 3. (C) Effect of NM on normal melanoma A2058 MMP-2 and MMP-9 expression: densitometric analysis

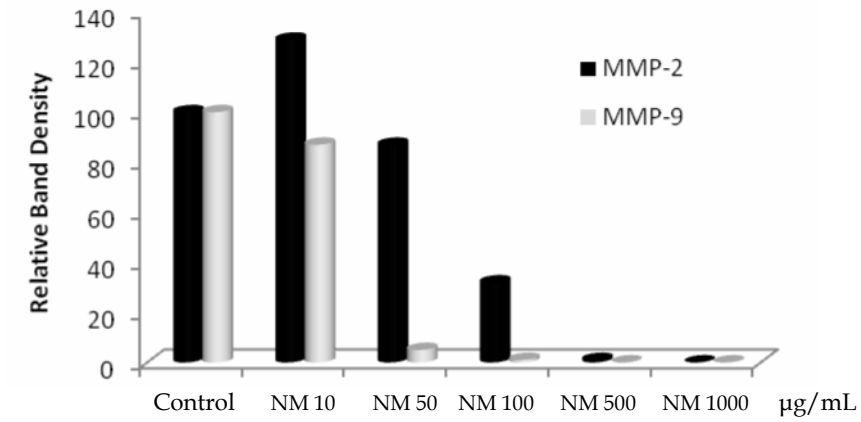


Fig. 3. (D) Effect of NM on PMA (100 ng/mL)-treated melanoma A2058 MMP-2 and MMP-9 expression: densitometric analysis

4.2 Matrigel invasion

Invasion studies were conducted using Matrigel (Becton Dickinson) inserts in 24-well plates. Suspended in medium, melanoma A2058 cancer cells were supplemented with nutrients, as specified in the design of the experiments 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 media from the wells were withdrawn. The cells on the upper surface of the inserts were gently scrubbed away with cotton swabs. The cells that had penetrated the Matrigel membrane and migrated onto the lower surface of the Matrigel were stained with hematoxylin and eosin and visually counted under the microscope.

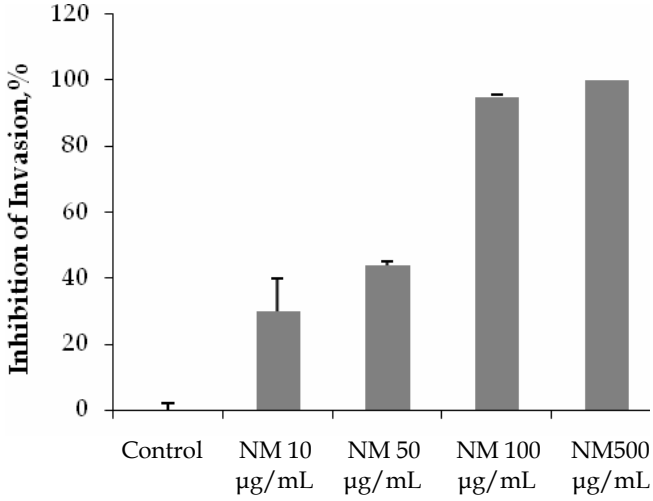


Fig. 4. Effect of NM on invasion of melanoma A2058 cells through Matrigel

Invasion of melanoma A2058 cells through Matrigel was inhibited by 30%, 44%, 95% and 100% by 10, 50,100, and 500 µg/mL of NM, respectively ($p<0.0001$), as shown in Figure 4 (Roomi et al., 2006b).

4.3 Cell migration: Scratch test

To study cell migration, a 2-mm wide single uninterrupted scratch was made from the top to bottom of culture plates of melanoma A2058 and B16FO cells grown to confluence. Culture plates were washed with PBS and incubated with NM in medium and tested at 0, 50, 100, 250 and 500 µg/mL, in triplicate at each dose for 24h. Cells were washed with PBS, fixed and stained with H&E and photomicrographs were taken. NM reduced cell migration in a dose-dependent manner in both cell lines, with complete block of A2058 at 500 µg/ml and B16FO at 250 µg/ml. Photomicrographs of the results for the scratch tests for melanoma A2058 (Figure 5) and B16FO (Figure 6) are shown below.

5. Micronutrient synergy inhibits cancer cell growth and apoptosis

ECM degradation is a prerequisite for cancer metastasis, but also it is essential to tumor growth and expansion and angiogenesis. Therefore, we investigated the effects of nutrient synergy on these important aspects of malignancy

5.1 Inhibition of tumor growth (xenografts)

The effect of dietary NM 0.5% on tumor growth was studied in athymic male nude mice using the model of melanoma A2058 and B16FO xenografts (Roomi et al., 2006b). Male athymic mice six weeks of age (n=12) were inoculated subcutaneously with 3 $x10^6$ A2058 cells in 0.2 mL PBS and 0.1 mL Matrigel. After injection, the mice were randomly divided into two groups; group A mice were fed regular Purina mouse chow and group B the regular diet supplemented with 0.5% NM (w/w). After four weeks, the mice were sacrificed and their tumors were excised and processed for histology. Dimensions (length and width) of tumors were measured using a digital caliper, and the tumor burden was calculated using the following formula: 0.5 x length x width. Mean weight of mice at initiation of study and termination of study did not differ significantly between the groups. NM supplementation demonstrated significant reduction in tumor weight (by 57%, $p<0.0001$ over control) and tumor burden by 31%, as well as decrease in vascularity.

Tissue samples were fixed in 10% buffered formalin, embedded in paraffin and cut at 4-5 microns for histological evaluation. After deparaffinization and appropriate epitope retrieval, the sections were incubated with primary antibody. Detection was by biotinylated goat anti-mouse antibodies followed by streptavidin conjugated to horseradish peroxidase with the use of diaminobenzidine as the chromogen. Polyclonal rabbit anti-human antibodies were used for MMP-9, MMP-2, VEGF, fibronectin and ki-67. Histological evaluation noted inhibition of MMP-9 and VEGF (an indicator of angiogenesis) secretion and mitotic index (ki-67) in mice fed the NM diet, as shown in Figure 7. There is a strong positive correlation between high ki-67 index and high-grade histopathology of neoplasms.

The effect of dietary NM 0.5% on tumor growth in athymic male nude mice was also studied using melanoma B16FO xenografts (Roomi et al, 2008). The nutrient-supplemented mice developed significantly smaller tumors (reduction in weight by 47%, $p=0.0002$). Reduction of tumor weight in melanoma A2058 and B16FO cell xenografts in nude mice is presented in Figure 8.

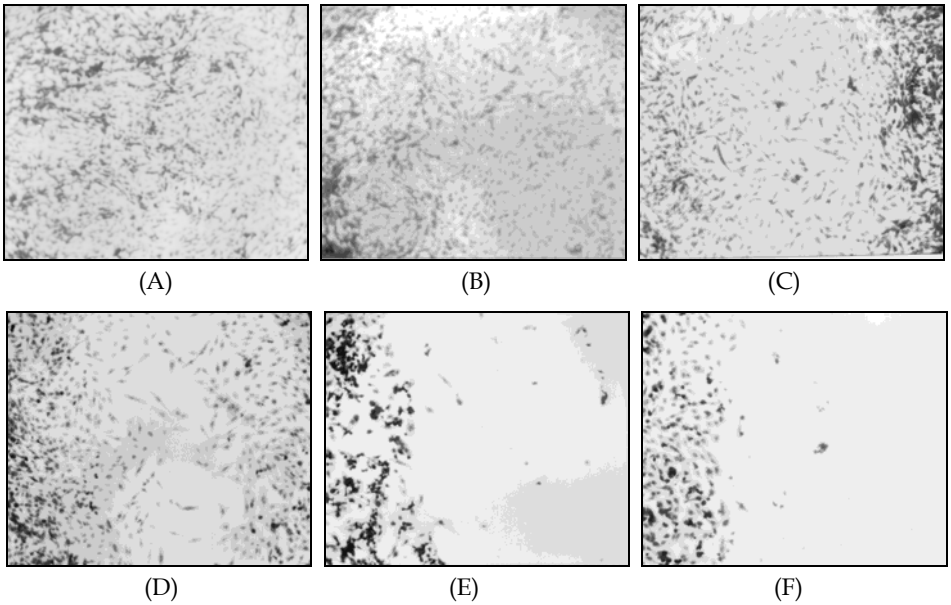


Fig. 5. Effect of NM on melanoma A2058 cell migration: scratch test: A – pre-scratch, B – control, C – NM 50 µg/mL, D – NM 100 µg/mL, E – NM 250 µg/mL, F- NM 500 µg/mL

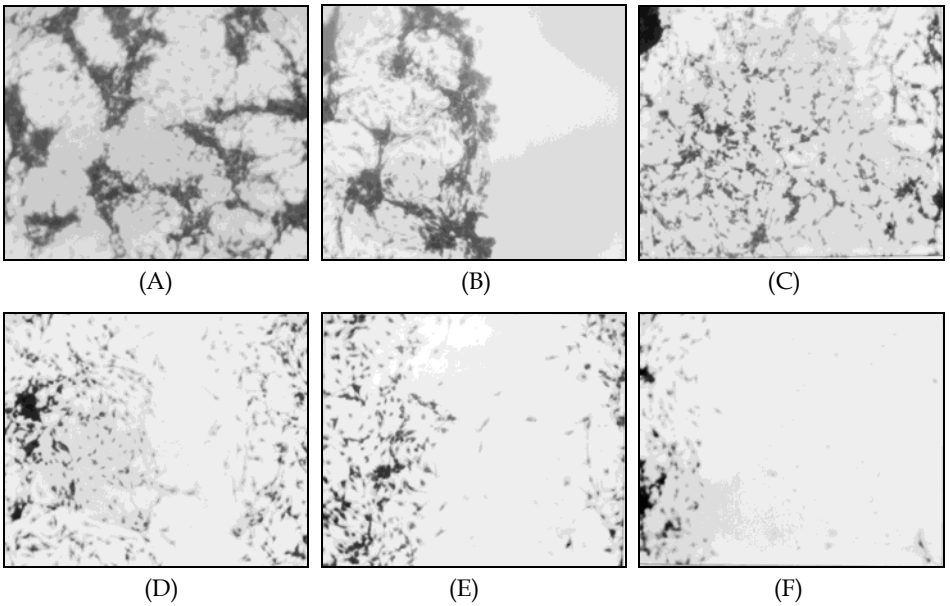


Fig. 6. Effect of NM on melanoma B16FO cell migration: scratch test: A – pre-scratch, B-scratch, C– control, D – NM 50 µg/mL, E – NM 100 µg/mL, F – NM 250 µg/mL

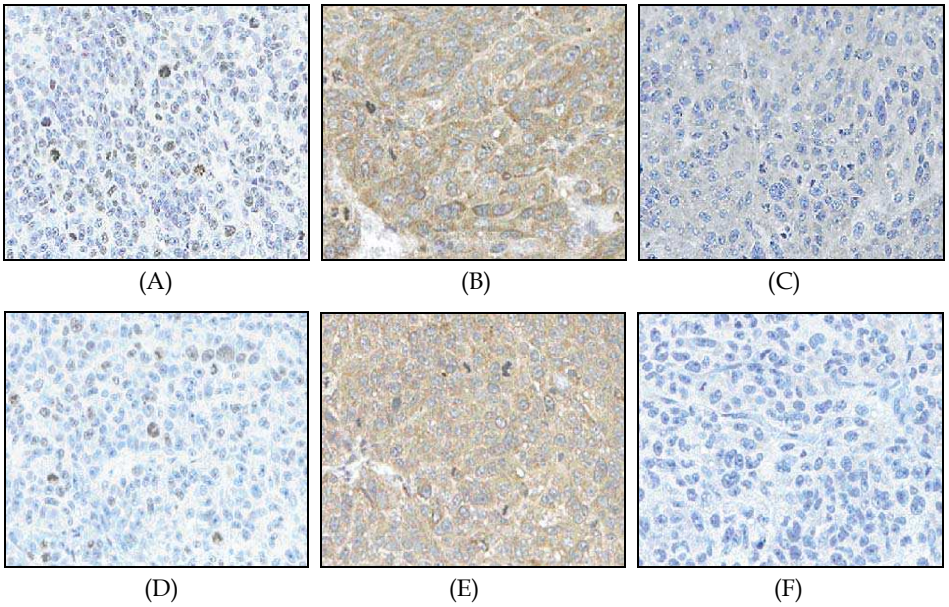


Fig. 7. Effect of NM on MMP-9, VEGF and ki-67 in A2058 xenograft tumors: A – Control Ki-67, B - Control MMP-9, C – Control VEGF, D – NM Ki-67, E – NM MMP-9, F – NM VEGF

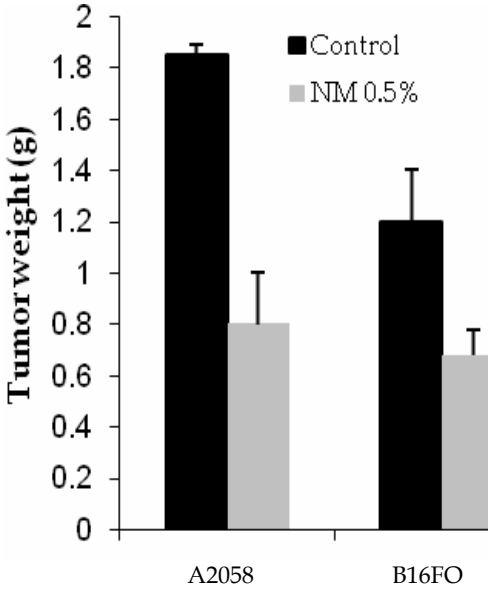


Fig. 8. Effect of supplementation with 0.5% NM on mean tumor weight of melanoma A2058 and B16FO xenografts in nude mice

5.2 Decreased cancer cell proliferation in the presence of nutrient synergy

We evaluated the effect of various concentrations of NM on the viability of melanoma A2058 and B16FO cells utilizing the MTT assay, a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide] (MTT) to a blue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells. This test is a good index of mitochondrial activity and thus of cell viability. NM had minimal (10%) antiproliferative effects on A2058 cells at 100 µg/mL, but at 500 µg/mL, inhibited cell viability by 64% ($p<0.0001$) (Roomi et al., 2006b). Melanoma B16FO cell proliferation was not inhibited up to 100 µg/ml, but at 500 and 1000 µg/ml, inhibited cell viability by 44% ($p=0.001$) (Roomi et al., 2006b). See Figure 9 for graphic representation of NM effect on melanoma B16 and A2058 cell proliferation.

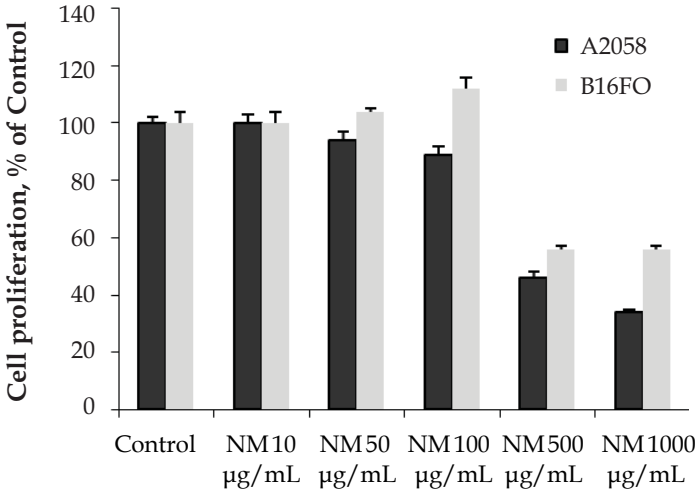


Fig. 9. Effect of NM on melanoma A2058 and B16FO cell proliferation

5.3 Induction of cancer cell apoptosis NM

We investigated pro-apoptotic effects of NM in both melanoma B16FO and A2058 cell lines utilizing the live-green caspases kit. To study the effect of NM on apoptosis of cancer cells, cells were grown to confluence, challenged with NM dissolved in media at 0, 100, 500, and 1000 µg/mL and incubated for 24 h. The cell culture was washed with PBS and treated with the caspase reagent as specified in the manufacturer's protocol (Molecular Probes Image-IT™ Live Green Poly Caspases Detection Kit 135104, Invitrogen). The cells were photographed under a fluorescence microscope and counted. Green-colored cells represent viable cells, while yellow orange represents early apoptosis and red, late apoptosis.

Dose-dependent induction of apoptosis in melanoma A2058 and B16FO cell lines was confirmed with NM challenge. Treatment of A2058 cells with NM demonstrated: 95.5% of cells were viable and 4.5% in apoptosis at 0 µg/mL NM; 0.7% were viable, 35.5% in early apoptosis and 63.8% in late apoptosis at 100 µg/mL NM; and 1.3% of cells were viable, 10.3% in early apoptosis, and 88.4% in late apoptosis at 500 µg/mLNM. Virtually all cells exposed to 1000 µg/mL NM were in late apoptosis. Photomicrographs of apoptotic cells and quantitative analysis of live, early and late A2058 apoptotic cells are shown in Figures 10 and 11.

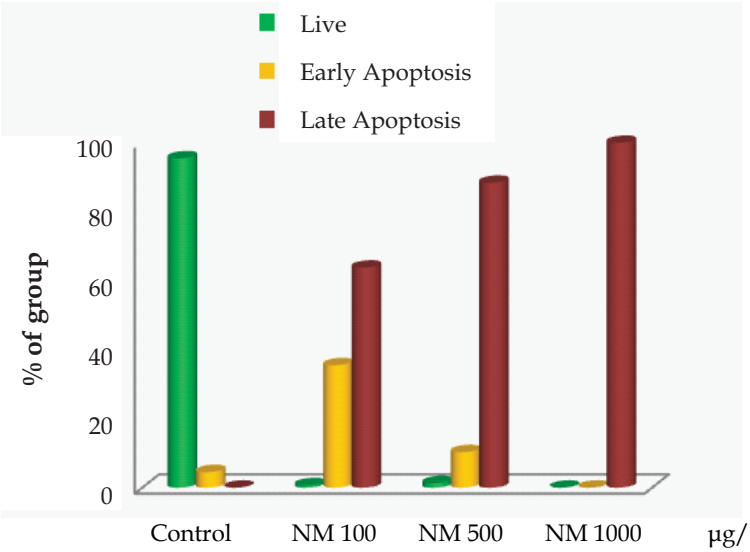


Fig. 10. Effect of NM on induction of melanoma A2058 apoptosis: quantitative analysis

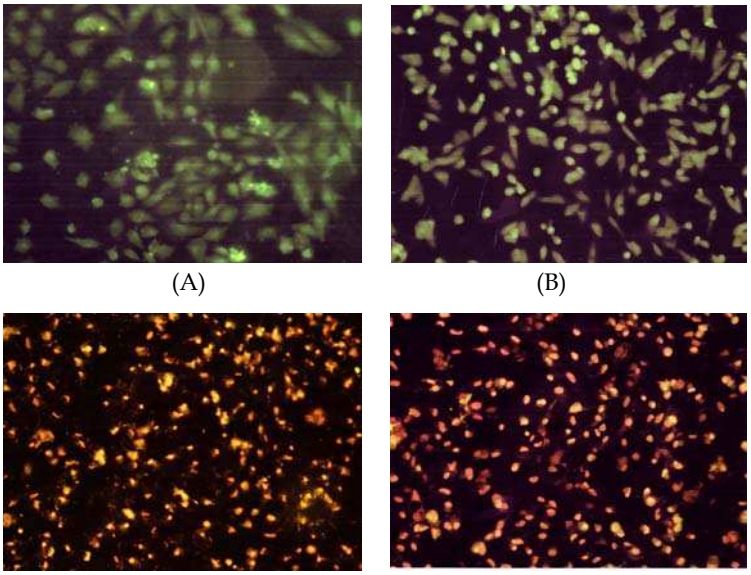


Fig. 11. Effect of NM on induction of melanoma A2058 apoptosis: photomicrographs
A – Control, B – NM 100 µg/mL, C - NM 500 µg/mL, D – NM 1000 µg/mL

B16FO cells treated with NM also shown dose-dependent apoptosis with slight induction of apoptosis (46% live, 33.3% early apoptosis and 20.6% late apoptosis) at 100 µg/mL NM,

moderate (15%live, 4.3% early apoptosis and 82.2% late apoptosis) at 500 µg/mL NM, and extensive apoptosis (9.6% live, 0.2% early apoptosis and 86.2% late apoptosis) at 1000 µg/mL NM, as shown in Figures 12 and 13 (Roomi et al., 2008).

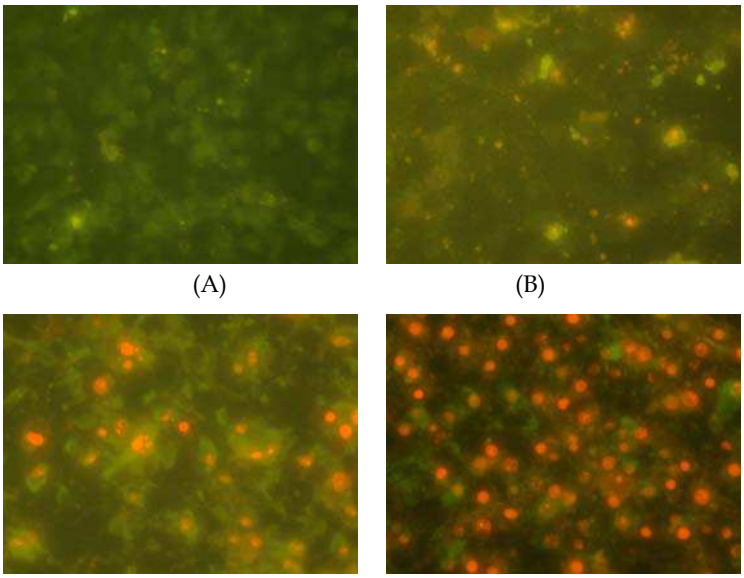


Fig. 12. Effect of NM on induction of melanoma B16FO apoptosis: photomicrographs: A – Control, B – NM 100 µg/mL, C - NM 500 µg/mL, D – NM 1000 µg/mL

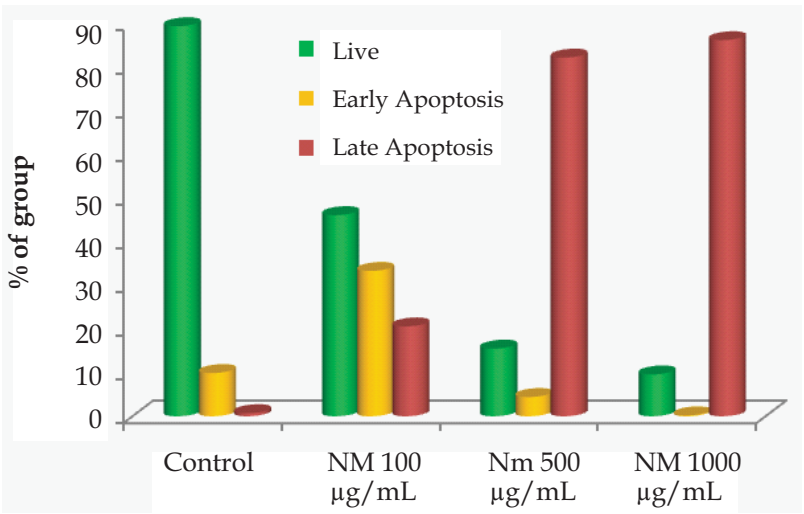


Fig. 13. Effect of NM on induction of melanoma B16FO cell apoptosis: quantitative analysis

5.4 Nutrient synergy modulates cancer cell cycle

We also studied the effect of NM on cell cycle in melanoma cells since deregulation of the cell cycle components may lead to tumor formation. Melanoma cells A-2058 were cultured in a 6-well plate at a concentration of 0.5 X 10^6 cells per well. The cells were incubated with NM at 0, 10, 100 and 1000 µg/mL for 24 hours and harvested. The cells were washed once with PBS and fixed in 70% ethanol. Cell pellets were suspended in 2 µL of 10 µg/ml RNase containing 0.5% Triton and the same volume of 20 µg/mL propidium iodide, followed by incubation in the dark at room temperature for 30 minutes. Cell fluorescence was measured in the Coulter EPIC Flow Cytometer.

Melanoma A-2058 cells exposed to NM showed dose-dependent increase of % of cells in the G-1 phase and decrease in G-2 and G-3 phases, indicating decrease in cells ready to undergo mitosis. See Figure 14.

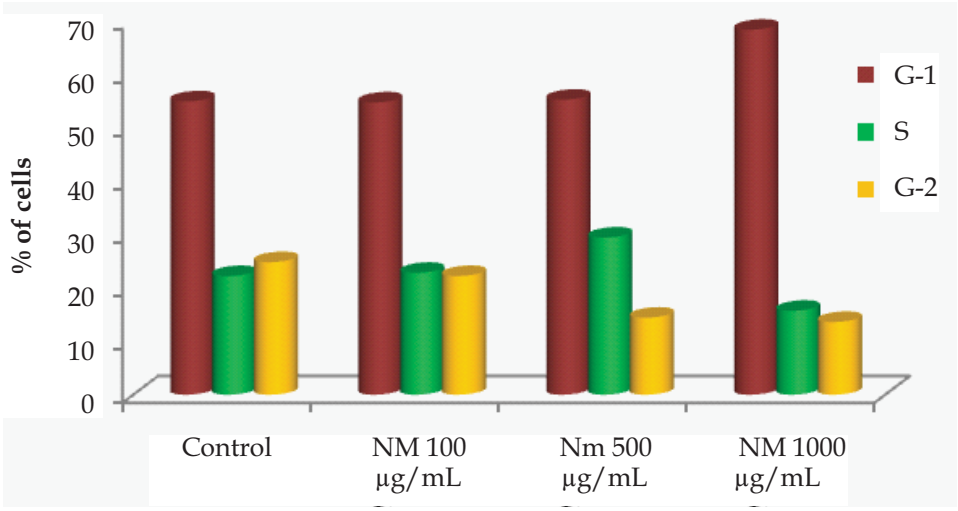


Fig. 14. Effect of NM on cell cycle of melanoma A-2058 cells

6. Conclusion

In conclusion, the results from these studies clearly indicate the strong anticancer potential of the micronutrient mixture based on its effective inhibition of melanoma B16FO and A2058 cells in vivo and in vitro. The NM showed profound inhibitory effects on melanoma B16FO pulmonary metastasis and hepatic metastasis and intrasplenic growth in C57BL/6 mice. Furthermore, tumor growth of melanoma A2058 and B16FO xenografts were significantly inhibited by dietary supplementation with NM. The in vitro studies supported these findings as they demonstrated inhibition of MMP-2 and MMP-9 secretion, cell migration, Matrigel invasion and cell proliferation, and induction of apoptosis. Furthermore, use of the nutrient mixture would not pose any toxic effect clinically, especially in the relevant doses, as in vivo safety studies demonstrate. During an in vivo study on possible toxicity from NM, we found that NM had neither adverse effect on vital organs (heart, liver, and kidney), nor on the associated functional serum enzymes (Roomi et al., 2003).

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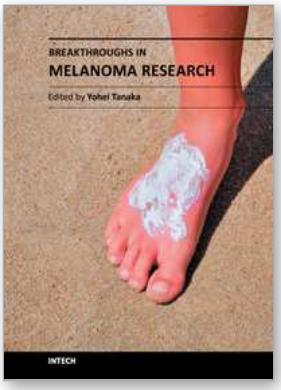

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Melanoma is considered to be one of the most aggressive forms of skin neoplasms. Despite aggressive researches towards finding treatments, no effective therapy exists to inhibit the metastatic spread of malignant melanoma. The 5-year survival rate of metastatic melanoma is still significantly low, and there has been an earnest need to develop more effective therapies with greater anti-melanoma activity. Through the accomplishment of over 100 distinguished and respected researchers from 19 different countries, this book covers a wide range of aspects from various standpoints and issues related to melanoma. These include the biology of melanoma, pigmentations, pathways, receptors and diagnosis, and the latest treatments and therapies to make potential new therapies. Not only will this be beneficial for readers, but it will also contribute to scientists making further breakthroughs in melanoma research.

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