WINNING THE GLOBAL FIGHT AGAINST CANCER: THE NUTRIENT SYNERGY APPROACH

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ABSTRACT

Consumption of plant-based diets has been shown to have a beneficial effect on cancer prevention and development. We have developed a combination of micronutrients containing lysine, proline, ascorbic acid and green tea extract (NM) that act synergistically to inhibit cancer development and its metastasis by strengthening collagen and connective tissues. NM has exhibited anticancer activity in vitro and in vivo in a number of cancer cell lines from various organ malignancies. In vitro studies demonstrated the effectiveness of NM in inhibition of cell proliferation, invasion, migration, MMPs, u-PA, angiogenesis, and up regulation of TIMPs and induction of apoptosis. In vivo, utilizing xenografts, NM significantly reduced the tumor size and tumor burden. NM also inhibited the growth of chemically-induced tumors in breast, skin and lung. Many cancers are often diagnosed at a later stage when metastasis has occurred, and standard treatment has failed to control its progress. Our studies on pulmonary, hepatic, testicular and peritoneal metastasis, using a mouse model, demonstrated significant reduction with NM treatment. Our results suggest that NM is an excellent candidate for therapeutic use in the treatment of a wide variety of cancers by inhibiting critical parameters such as proliferation, invasion, metastasis, and angiogenesis and by inducing apoptosis.

INTRODUCTION

Cancer, an abnormal and uncontrolled growth of malignant cells, is the second leading cause of death in the Western world, affecting people of all ages. Cancer can originate from almost any site in the body and then spreads to other parts of the body. Almost 1.4 million new cases of cancer are diagnosed each year (NCI, 2010), and there are more than 100 different types of this disease. The risk of developing cancer during one’s lifetime is 50% for men and 33% for women. The most prevalent type of cancer in men is prostate cancer and for women it is breast cancer. In both genders lung and colorectal cancer are prevalent.

Radiation, chemicals and viruses have been recognized as cancer-causing agents in human and many animal species. Although there is a great diversity in the nature of these agents, the end cellular response is always the same - the production of cancer cells. The transformation of normal cells to cancerous cells is a complex and stepwise process including initiation, promotion and progression.

Standard cancer treatments, utilizing a combination of surgery, multiple chemotherapeutic agents and ionizing radiation, have been largely unsuccessful. For example, a review of clinical trials conducted between 1990 and 2004 on 22 types of cancer noted that chemotherapy merely increased 5-year survival by 2.1% (Morgan et al., 2004). Chemotherapy is associated with poor outcomes secondary to its severe toxicity, immune involvement, and genotoxicity, giving rise to new cancers, as well as development of drug resistance by cancer cells. Furthermore, standard
cancer treatments are costly and have led to the immense increased cost of healthcare. Thus, there is a need for defining new biological targets and applying non-toxic therapeutic solutions.

In the search for effective solutions to cancer, the work of Dr. Rath provides a new perspective in the therapeutic use of essential nutrients to control the growth of cancer and its metastasis. Dr. Rath’s approach has been to develop strategies to inhibit cancer development, progression and metastasis using naturally occurring nutrients (Rath and Pauling, 1997). Lysine and proline are natural amino acids, the building blocks of collagen and elastic fibers that stabilize connective tissues. In addition, they prevent the degradation of collagen by blocking sites where digestive enzymes attach themselves. Vitamin C is essential for the production of collagen and is a scavenger of free radicals that protects cells from damage. Epigallocatechin gallate (EGCG) is a potent polyphenolic fraction of green tea that exerts anti-proliferative, antimutagenic and anti-neoplastic activity. It was postulated that the combination of these nutrients exerts potent synergistic anti-cancer activity. These nutrients are the natural inhibitors of the extracellular matrix and are essential in reinforcing and strengthening the connective tissue that surrounds cancer cells (tumor “encapsulating effect”). Based on this prediction, the Nutrient Mixture (NM) was formulated by the Dr. Rath Research Institute. (See Table 1 for composition of NM.)

NM has been studied in vitro and in vivo using various cancer cell lines to test its effects on the hallmarks of cancer, including tumor cell proliferation, growth, invasion, metastasis, angiogenesis and apoptosis effects. NM was found to inhibit tumor growth/proliferation, invasion, metastasis and angiogenesis and to induce apoptosis.

**EFFECT OF THE NUTRIENT MIXTURE ON CANCER CELL PROLIFERATION AND TUMOR GROWTH**

**IN VITRO EFFECT OF NM ON CANCER CELL PROLIFERATION**

We first evaluated the effect of NM on proliferation in vitro on a number of cancer cell lines. Forty-five different cancer lines were selected on the basis of organ malignancies that included carcinomas, sarcomas, leukemia and five murine cancer cell lines. Inhibition of proliferation was confirmed in a variety of human cancer cell lines (Niedzwiecki et al., 2010). The effect of NM on human melanoma cell line A2058, a representative example, is shown in Figure 1. NM had a slight effect at 10 and 50 µg/ml and a potent effect at 500 and 1000 µg/ml (Roomi et al., 2011).

**TUMOR GROWTH IN VIVO: XENOGRAFT STUDIES**

The findings in vitro were consistent with in vivo experiments. NM inhibited the growth of xenografts using various cancer cell lines in nude mice. Tumors were induced by injection of cancer cells and mice were randomly divided into two groups and were fed either a standard diet or a diet enriched with 0.5% NM. After four weeks, the mice were sacrificed and their tumors were excised and processed for histology. NM showed reduced tumor growth with the following cell lines: colon cancer cells (HCT-116) by 63%, melanoma cells (A2058) by 57%, prostate cancer cells (PC-3) by 53%, osteosarcoma cells (MNNG) by 53%, breast cancer (MDA-MB231) by 27%, and neuroblastoma cells by 25% (Niedzwiecki et al., 2010). Representative tumors from the NM and Control diet groups and a graph of the relative tumor weight in groups are shown for
the breast cancer MDA-MB-231 xenograft study in Figure 2.  NM exhibited not only a significant reduction in tumor size, but also in tumor weight (Figure 2).

**EFFECT OF NM ON CHEMICALLY-INDUCED TUMORS IN MAMMARY GLAND, LUNG AND SKIN**

Based on above encouraging results, in the next series of studies we determined the efficacy of NM in inhibiting the carcinogenic process induced by chemicals in different organs. Accordingly, we studied the inhibitory effect of NM on N-nitroso-N-methylurea (MNU), urethane and 7, 12-dimethylbenzen(a)anthracene (DMBA)-induced carcinogenic process in the mammary gland, lung and skin respectively.

Breast tumors induced in female rats using MNU were significantly reduced by NM dietary supplementation (Niedzwiecki et al., 2010). Incidence of tumors was reduced from 19 tumors in the control group to 6 in NM group. NM also significantly decreased the tumor weight by 78% and tumor burden by 60.5%, as shown in Figure 3. NM supplemented mice also demonstrated significant reduction in the number of tumors induced by urethane compared to the Control group mice, as shown in Figure 4A and in the number of skin tumors (papillomas) induced by DMBA compared to the Control group, as shown in Figure 4B (Niedzwiecki et al., 2010). Results from these in vivo carcinogenic studies clearly indicate that NM inhibits cancer development in three different organs induced by three different chemical carcinogens.

**EFFECT OF THE NUTRIENT MIXTURE ON CANCER CELL INVASION THROUGH MATRIGEL**

Cancer is capable of spreading through the body by two mechanisms: invasion and metastasis. Invasion refers to the direct migration and penetration by cancer cells into the neighboring tissues.

NM inhibited invasion of several human cancer cell lines through Matrigel in a dose dependent fashion. The following illustrates a typical invasion experiment using fibrosarcoma cells HT-1080. Invasion studies were conducted using Matrigel (Becton Dickson) matrix-coated 9-mm cell culture inserts (pore size 8um) set in 24-well plates, using a modified Boyden Chamber. The invasion of melanoma cells through Matrigel was inhibited by 40%, 50%, 70% and 100% by 10, 100, 200 and 1000 µg/ml NM, respectively (Figures 5A, 5B) (Roomi et al., 2011). Similarly, NM inhibited Matrigel invasion of a number of human and murine cancer cell lines.

**EFFECT OF NM ON IN VITRO MIGRATION OF TUMOR CELLS**

NM reduced migration of fibrosarcoma HT-1080 cells in a dose dependent fashion in a 2-mm wide single uninterrupted scratch from top to bottom at near confluent culture plate (Niedzwiecki, 2010). Similar results were obtained using other cancer cells.

Having demonstrated that NM inhibits invasion and migration of tumor cells, the next series of experiments were designed to determine whether NM would also inhibit metastasis of tumor cells. Both in vivo and in vitro experiments were carried out.
EFFECT OF THE NUTRIENT MIXTURE ON METASTASIS

Metastasis refers to the ability of cancer cells to penetrate into blood vessels and the lymphatic system, circulate via the blood stream and be transported to distal organs, where they grow and form separate colonies. Metastatic potential and invasiveness of cancer are attributed to the up-regulation of matrix metalloproteinases (MMPs) and urokinase plasminogen activator (u-PA) (Chambers et al., 1997, Kleiner et al., 1999, Liotta et al, 1980, Stetler-Stevenson, 2001). Proteolytic activities of MMPs are inhibited by specific inhibitors, tissue inhibitors of metalloproteinases (TIMPs). Thus, a critical determinant of net proteolytic degradation is the balance between MMP and TIMP levels.

MMPs are a class of zinc-dependent neutral proteinases encoded by a multi-gene family. Secretion of MMP-2 and MMP-9 is elevated in several types of human cancers and is associated with poor prognosis. The activity of these enzymes correlates with the aggressiveness of tumor growth and invasiveness of cancer. We tested the effect of NM on several different cancer cell lines based on organ malignancies, including carcinomas, sarcomas and leukemia. Based on the MMP-2 and MMP-9 secretion the various cancer cell lines could be categorized into three groups: 1) those secreting only MMP-2; 2) those secreting MMP-9 only; and 3) those secreting both MMP-2 and MMP-9. NM inhibits the expression of MMPs in all three different classes in a dose-dependent fashion (Roomi et al., 2010). The effect of NM on fibrosarcoma HT-1080, a representative of a cell line that basally secretes both MMPs, is shown in Figure 6. NM has also been shown to inhibit u-PA and up regulate TIMPs in a number of cancer cell lines.

IN VIVO INHIBITION OF PULMONARY AND HEPATIC METASTASIS OF MELANOMA B16F0 CELLS IN C57BL/6 MICE

We also investigated the effect of NM on lung and hepatic metastasis. Pulmonary metastasis of melanoma B16F0 cells in C57BL/6 female mice injected via the tail vein was reduced by 63% in mice supplemented with the NM diet (Roomi et al., 2006). We also investigated the effect of NM on hepatic metastasis of B16FO melanoma cells in C57BL/6 mice. B16FO melanoma cells (10⁶) were injected into the spleen and divided into two groups. The mice supplemented with NM not only showed less tumor growth in the spleen compared to the control, but also reduced metastasis to the liver. Hepatic metastasis in NM supplemented mice was reduced by 55% compared to the control (based on mean liver weights of the group), as shown in Figures 8A-C (Roomi et al., 2008).

EFFECT OF THE NUTRIENT MIXTURE ON ANGIOGENESIS

Angiogenesis is the formation of new capillaries from existing blood vessels. It is considered to be a fundamental process in physiological and pathological conditions. It is also necessary for tumor growth, invasion and metastasis. Angiogenesis not only allows the tumor to increase in size, but also provides a route for metastasis to distal sites in the body. A tumor mass less than 0.5 mm in diameter can survive by receiving O₂ and nutrients by diffusion. Any increase in tumor mass beyond 0.5 mm requires angiogenesis. We used several in vitro and in vivo experimental models to determine if NM exhibits anti-angiogenic effects. NM demonstrated significant antiangiogenic activity, as demonstrated in vivo by the chorioallantoic membrane (CAM) assay in chick embryos and by basic fibroblast factor (bFGF)-induced vessel growth in mouse Matrigel plug assay (Niedzwiecki et al., 2010). In addition, in vitro, NM decreased the
expression of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), angiopoietin-2, bFGF, platelet-derived growth factor (PDGF) and transforming growth factor (TGFβ-1) by U2OS osteosarcoma cells in vitro.

Xenograft studies using human osteosarcoma MNNG-HOS showed that the NM supplemented nude mice developed significantly smaller tumors by 53% and were less vascular compared to the control (Figure 9). Immunohistochemical analysis showed decreased VEGF staining, an indicator of angiogenesis, in NM treated group compared to the control group. MMP-9 expression was also lower in the NM supplemented group compared to the corresponding control group.

EFFECT OF THE NUTRIENT MIXTURE ON APOPTOSIS

We investigated whether the underlying anti-tumor effect of NM was due to apoptosis. Apoptosis, also known as programmed cell death, is a complex process that occurs in several pathological processes. Normal cells undergo a regular cycle of generation and death. However, the normal cycle is absent in cancer cells, making cancer cells immortal. This makes cancer cells especially dangerous since each cell has the potential to divide endlessly. Apoptosis is distinguished from necrosis by characteristic morphological and biochemical changes. These changes include the compaction and fragmentation of nuclear chromatin, shrinkage of the cytoplasm and loss of membrane asymmetry. Several assays have been developed based on apoptotic properties. A distinctive feature of early stages of apoptosis is the activation of caspase enzymes. The caspase family of cysteine-aspartic acid specific protease is emerging as a central executioner of apoptosis (e.g. caspases-3, -7, -8, -9, -10).

Using a Live Green Caspases Detection Kit (Molecular Probes, I35104), we studied apoptosis in a number of cancer cell lines in vitro. The cancer cell lines were cultured in their recommended medium and treated with NM in different concentrations: 0, 100, 250, 500 and 1000 µg/ml. With Live Green Poly Caspases, the cells were photographed under a fluorescence microscope and counted. Green-colored cells represented viable cells, yellow and orange cells early apoptosis, and red cells late apoptosis. Dose-dependent induction of apoptosis in a variety of cancer cells lines was confirmed with NM challenge. Quantitative analysis of live, early and late apoptotic cells in rhabdomyosarcoma is shown in Figure 10.

CONCLUSION

In brief the results of the present study clearly indicate that NM inhibits all the hallmarks of cancer development. For example, NM was shown to (a) inhibit cell proliferation in a wide variety of cancer cells, (b) inhibit the growth of cancer cells implanted as xenografts in athymic mice, (c) inhibit cancer development in three different organs, the mammary gland, the lung and the skin induced by three different chemical carcinogens, (d) inhibit secretion of MMPs by cancer cells, (e) inhibit migration and invasion of cancer cells, (f) inhibit angiogenesis using both in vitro and in vivo models, (g) inhibit metastasis, and (g) induce apoptosis in a number of cancer cell lines. Thus our results suggest that the nutrient mixture is highly effective in inhibiting all important mechanisms in cancer development and offers unique benefits in fighting cancer worldwide.
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<tr>
<th>Nutrient</th>
<th>Molar Concentration (in 100 µg/ml solution)</th>
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<tr>
<td>Vitamin C</td>
<td>90 µM</td>
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<tr>
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<td>L-Proline</td>
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<td>Manganese</td>
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Figures

Figure 1 – Effect of NM on melanoma A2058 cell proliferation: MTT assay

Figure 2 – Effect of NM on breast cancer (MDA-MB-231) xenograft tumor growth in female nude mice: 2A – Representative tumors from groups; 2B – Mean tumor weight of groups
Figure 3 - Effect of NM on total tumor weight and tumor burden in MNU-induced tumors in Sprague-Dawley mice

3A - Effect of NM supplementation on tumor burden

3B - Effect of NM supplementation on tumor weight

Figure 4A - Effect of NM supplementation on tumor weight

Figure 4B - Effect of NM supplementation on tumor burden

Average = 18.3
Tumor burden decreased by 60.5%

Figure 4A - Effect of NM on (4A) lung tumors induced by urethane in male A/J mice: histological lung sections representative of groups: Control diet and NM 0.5% supplemented diet and effect of NM on (4B) DMBA-induced papillomas in female SENCAR mice: photographs of representative mice in each group: Control diet and NM 0.5% diet

Figure 5A - Effect of NM on inhibition of Matrigel invasion by fibrosarcoma HT-1080 cells
Figure 5B – Effect of NM on inhibition of Matrigel invasion by fibrosarcoma HT-1080 cells - photomicrographs

Figure 6 – Effect of NM on fibrosarcoma HT-1080 MMP-2 and MMP-9 secretion

Legend: 1 -Markers, 2-Control, 3-7 NM 10, 100, 200, 500, 1000 µg/ml
Figure 8– Effect of NM on lung and hepatic metastasis of melanoma B16FO cells injected into C57BL/6 female mice fed control or NM 0.5% supplemented diet: A – lung, B – spleen, C-liver

Figure 9 – Effect of NM 0.5% diet on osteosarcoma MNNG-HOS cell xenografts in athymic nude mice

Figure 10 – Effect of NM on induction of apoptosis in rhabdomyosarcoma cells:
REFERENCES


