

## Inhibition of 7,12-dimethylbenzanthracene-induced skin tumors by a nutrient mixture

M. W. Roomi · N. W. Roomi · T. Kalinovsky · V. Ivanov · M. Rath · A. Niedzwiecki

Received: 17 December 2007 / Accepted: 27 December 2007 / Published online: 18 January 2008  
© Humana Press Inc. 2008

**Abstract** The annual incidence of all forms of skin cancer, the most common of all human cancers, is increasing yearly. A unique nutrient mixture (NM) was shown to exhibit anticancer activity in vivo and in vitro. We examined the effect of NM on the development of skin cancer induced by 7,12-dimethylbenzanthracene (DMBA) in female SENCAR mice by a complete carcinogenesis protocol. Mice ( $n = 55$ ) were divided into four groups and carefully shaved on dorsum. After 2 days, the mice in Groups 1 ( $n = 10$ ), 3 ( $n = 20$ ), and 4 ( $n = 20$ ) were treated topically with 100 nM DMBA in 0.2 ml of acetone twice a week for 4 weeks; Group 2 ( $n = 5$ ), the control group, was treated with acetone 0.2 ml. Groups 1 and 2 were fed the regular diet. Group 3A ( $n = 10$ ) was fed a diet containing 0.5% NM from the day of DMBA treatment and 3B ( $n = 10$ ) the regular diet and received NM (75 mg in 0.4 ml of 1:1 acetone/water) topically to the shaved area 15 min before DMBA application twice a week for 4 weeks. Group 4 mice were fed a diet containing 0.5% NM for 2 weeks prior to the application of DMBA and then divided into two groups: 4A ( $n = 10$ ) was fed the 0.5% NM diet as in 3A, and 4B ( $n = 10$ ) the regular diet as described for 3B. Body weight and diet consumption of the mice were monitored and the skin tumors (papillomas) were counted and recorded. Ten weeks thereafter the mice were euthanized, skinned, and tumors were processed for histology. NM significantly ( $P < 0.0001$ ) inhibited DMBA-induced skin tumor multiplicity by 59, 62, 69, and 86% in NM-treated Groups 3A, 3B, 4A, and 4B, respectively. These results suggest that NM

has strong potential as a useful therapeutic regimen for skin cancer by significantly inhibiting the incidence and tumor multiplicity of DMBA-induced skin tumors.

**Keywords** Papillomas · DMBA · Skin cancer · Squamous cell carcinoma · Nutrients · SENCAR mice

### Introduction

Skin cancer, the most common of all human cancers, accounts for half of all cancers diagnosed and is reported to be increasing annually. Squamous cell carcinoma (SCC) is the second most common form of skin cancer with 1,000,000 new cases estimated in 2007 in the USA and closely associated with sun exposure and chemical carcinogens [1]. It is widely accepted that SCCs, which are responsible for the majority of non-melanoma skin cancer-related deaths, result from the accumulation of genetic alterations [2].

Skin tumors can be effectively induced in mice by the repetitive application of a carcinogen. Slaga et al. [3] proposed that the skin tumor initiation by chemical carcinogens, such as 7,12-dimethylbenz[a]-anthracene (DMBA), is probably an irreversible stage that involves a somatic mutation, mainly in the *Ha-ras* oncogene. In contrast to the initiation stage, the promotion stage is reversible and requires a series of applications to induce benign skin tumors. The progression stage appears to involve the accumulation of additional genetic changes in cells, which form skin papillomas that can be considered as a clone of initiated cells.

SENCAR mice were reported to be the most sensitive mouse group to carcinogens tested by Slaga et al. [4]. Skin tumor promotion and progression stages are characterized by selective inflammation and sustained

M. W. Roomi · N. W. Roomi · T. Kalinovsky · V. Ivanov · M. Rath · A. Niedzwiecki (✉)  
Oncology Division, Dr. Rath Research Institute, 1260 Memorex Drive, Santa Clara, CA 95050, USA  
e-mail: author@drath.com

hyperplasia, differentiation, alterations, and genetic instability of initiated cells developing into papillomas and carcinomas [3, 5]. Mice treated topically with tumor promoters have demonstrated that the histologically sustained cellular hyperplasia plays a critical role in tumor promotion [6].

Rath and Pauling [7] defined common pathomechanisms for all cancers, the destruction of extracellular matrix (ECM) as a precondition for cancer cell invasion, growth, and metastasis and suggested intervention through natural inhibitors of plasmin-induced proteolysis, such as lysine and its analogs. Cancer cells form tumors and spread by degrading the ECM through various matrix metalloproteinases (MMPs). The activity of these enzymes correlates with the aggressiveness of tumor growth and invasiveness of the cancer. Rath and Pauling postulated that nutrients such as lysine and ascorbic acid could act as natural inhibitors of ECM proteolysis and, as such, have the potential to modulate tumor growth and expansion. These nutrients can exercise their antitumor effect through the inhibition of MMPs, and in addition, by strengthening of connective tissue surrounding cancer cells through their effect on collagen synthesis. These two processes are essential for a tumor encapsulating effect. MMPs and constituents of ECM play a critical role in neoplastic invasion and metastasis.

We have developed strategies to inhibit cancer development and its spread using naturally occurring nutrients such as lysine, proline, ascorbic acid, and green tea extract (nutrient mixture, NM), which have exhibited synergistic anticancer activity *in vivo* and *in vitro* in a number of cancer cell lines, including human and murine melanoma cells lines [8, 9], inhibiting cancer cell growth, MMP secretion, invasion, metastasis, and angiogenesis. The present study examines the *in vivo* effect of NM on the development of skin cancer induced by DMBA in female SENCAR mice by a complete carcinogenesis protocol.

## Methods and materials

### Materials

The DMBA obtained from Sigma Chemical Co. (St. Louis, MO, USA) was dissolved in acetone to 100 nM in 0.2 ml acetone. Stock solution of the NM was composed of the following in the ratio indicated: Vitamin C (as ascorbic acid and as Mg, Ca, and palmitate ascorbate) 700 mg; L-lysine 1,000 mg; L-proline 750 mg; L-arginine 500 mg; *N*-acetyl cysteine 200 mg; standardized green tea extract

(80% polyphenol) 1,000 mg; selenium 30 µg; copper 2 mg; manganese 1 mg.

### Animals

Female SENCAR mice, ~6 weeks of age on arrival, were purchased from National Cancer Institute (Frederick, MD, USA) and maintained in microisolator cages under pathogen-free conditions on a 12-h light/12-h dark schedule for a week. All animals were cared for in accordance with institutional guidelines for the care and use of experimental animals.

### Experimental design

We studied the development of skin cancer induced by DMBA in female SENCAR mice using a complete carcinogenesis protocol based on work by Hanausek et al. [10]. After 1-week of isolation, the dorsal area of the 6-week-old female SENCAR mice ( $n = 55$ ) was carefully shaved with an electric clipper to avoid cuts and bruises. The mice were then divided into groups: 1–4. After 2 days, the mice in Groups 1 ( $n = 10$ ), 3 ( $n = 20$ ), and 4 ( $n = 20$ ) were treated topically with 100 nM DMBA in 0.2 ml of acetone twice a week for 4 weeks, while Group 2 ( $n = 5$ ) mice received only acetone 0.2 ml topical treatments. Groups 1 and 2 mice were fed the regular diet. Group 3 mice were further divided into two sub-groups: 3A and 3B. Group 3A ( $n = 10$ ) mice were fed a regular diet containing 0.5% NM from the day of DMBA treatment and Group 3B ( $n = 10$ ) mice were fed the regular diet and received NM (75 mg in 0.4 ml of 1:1 acetone/water) topically to the shaved area 15 min before DMBA application twice a week for 4 weeks. Group 4 mice were fed a regular diet supplemented with 0.5% NM for 2 weeks prior to the application of DMBA. At this time, the mice were further divided into two groups. Group 4A ( $n = 10$ ) was fed the 0.5% NM diet as in 3A, and 4B ( $n = 10$ ) the regular diet as described for 3B (see Table 1 for a summary of the interventions). Body weight and diet consumption of the mice were monitored and the skin tumors (papillomas) were counted and recorded. Ten weeks thereafter the mice were euthanized, skinned, and tumors were processed for histology.

### Histology

Tissue samples were fixed in 10% buffered formalin. All tissues were embedded in paraffin and cut at 4–5 µm. Sections were deparaffinized through xylene and

**Table 1** Summary of interventions

Groups	Topical DMBA 100 nM in 0.2 acetone 2×/week × 4 weeks	Pretreatment 0.5% NM in diet 2 weeks prior to DMBA	Diet—regular or 0.5% NM supplemented	Topical NM 75 mg/0.4 ml prior to DMBA treatment
1 ( <i>n</i> = 10)	DMBA	None	Regular	None
2 ( <i>n</i> = 5)	None	None	Regular	None
3A ( <i>n</i> = 10)	DMBA	None	0.5% NM	None
3B ( <i>n</i> = 10)	DMBA	None	Regular	Topical NM
4A ( <i>n</i> = 10)	DMBA	Pre-treatment	0.5% NM	None
4B ( <i>n</i> = 10)	DMBA	Pre-treatment	Regular	Topical NM

graduated alcohol series to water and stained with hematoxylin and eosin for evaluation using a standard light microscope.

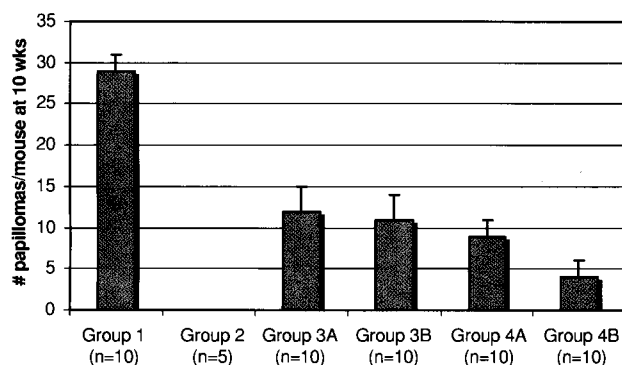
### Statistical analysis

The results were expressed as means  $\pm$  SD for the groups. Data were analyzed by independent sample “*t*” test.

## Results

### Tumor incidence and multiplicity

Nutrient mixture treatment significantly ( $P < 0.0001$ ) inhibited DMBA-induced skin tumor multiplicity (number of tumors per mouse) over the control group (Group 1) by 59, 62, 69, and 86% in Groups 3A, 3B, 4A, and 4B, respectively, as shown in Fig. 1. Gross photographs of mice with papillomas representative of each group are shown in



**Fig. 1** Effect of NM on multiplicity of DMBA-induced papillomas in female SENCAR mice at 10 weeks. NM significantly ( $P < 0.0001$ ) inhibited DMBA-induced skin tumor multiplicity by 59, 62, 69, and 86% in Groups 3A, 3B, 4A, and 4B, respectively. Topical NM treatment prior to DMBA application was the most effective treatment in inhibiting tumors, followed by 2-week pretreatment with diet enriched by 0.5% NM and treatment with NM 0.5% during the study

Fig. 2. Tumor incidence (number of mice with tumors) was decreased over the control by 20% in Groups 3A, 3B, and 4A and by 50% in Group 4B. Two-week pretreatment with a diet enriched by 0.5% NM was the most effective treatment in inhibiting tumors, followed by topical NM treatment prior to DMBA application, and with NM 0.5% supplemented diet during the study. The number of DMBA-induced papillomas in female SENCAR mice increased from 5 to 8 weeks and was significantly higher for the control group than the NM-treated groups, as shown in Fig. 3.

### Histopathology

Skin lesions included papillary proliferative lesions, both papillomas and keratocanthomas, with associated varying degrees of epidermal hyperplasia and generally severe dermal thickening with fibrosis and mild to moderate infiltration with inflammatory cells (Fig. 4). In the control group of mice (Group 1), not only were there significantly more papillomas, but also the incidence of early to well-developed SCCs with areas of ulceration was high, as shown in Fig. 4b–f. In mice receiving dietary NM only post DMBA application (Groups 3A and 3B), the papilloma incidence was reduced over the control, but histologically showed inflammation, infiltration, and SCC, as shown in Fig. 4g–k. Among the DMBA-treated mice, the mice that underwent dietary pretreatment with NM combined with topical treatment with NM (Group 4B) or dietary NM during the study (Group 4A), demonstrated similar pathological changes as Group 3, with hyperplastic papillomatous lesions without the evidence of infiltration and keratoacanthomas, as seen in Fig. 4l.

Mean body weights of SENCAR mice in different groups did not differ significantly neither at the beginning nor at the termination of the study, ranging at termination between 36.1 and 39.8 g. Consumption of food and water did not differ among the groups.

