UTILIZATION OF A PRECLINICAL MODEL FOR CHEMOPREVENTION OF ESOPHAGEAL CANCER EMPLOYING A FOOD-BASED AND SINGLE-AGENT APPROACH

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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ABSTRACT

Cancer incidence estimates for 2003 indicate that 18% of all cancers diagnosed originate in the aerodigestive tract, including the larynx, lung and bronchus, oral cavity, pharynx and esophagus. Specifically esophageal squamous cell carcinoma represents over 90% of all esophageal cancer cases. It is the 8th leading cause of cancer and the 5th leading cause of cancer death. Our laboratory has evaluated the chemopreventive potential of certain foods and a variety of single agents, including food-derived and synthetic compounds, in animal models of lung, colon and esophageal cancer. The goal of these studies was to determine the efficacy of food based and single agents for future use in human chemoprevention trials in individuals at high risk for esophageal cancer. Certain foods, particularly fruits and vegetables, contain a number of complex mixtures that possess preventative properties which may be active at multiple stages of carcinogenesis and/or have additive or synergistic effects. Following a food-based approach for cancer chemoprevention, we initially conducted a study to determine if a freeze-dried preparation of blueberries would inhibit N-nitrosomethylbenzylamine (N MBA)-induced esophageal tumorigenesis. A single-agent strategy for cancer chemoprevention typically involves the use of an agent which targets a specific process in carcinogenesis. Earlier studies have
demonstrated that elevated polyamines may play an important role in the
development of tumors in the human esophagus. Based on our findings, our
second study evaluated the effect of difluoromethylornithine (DFMO), an
irreversible inhibitor of ornithine decarboxylase, on post-initiation events in
NMBA-induced rat esophageal tumorigenesis. Since black raspberries are
effective at inhibiting tumors in both during the initiation and promotion /
progression stages of carcinogenesis, our third study evaluated whether black
raspberries might also exhibit therapeutic effects against esophageal cancer.
The results of our studies indicated that freeze-dried blueberries were ineffective
as inhibitors of esophageal tumorigenesis in the rat esophagus. Although the
black raspberries may be effective during initiation and promotion / progression
stages of carcinogenesis, they did not appear to exhibit any therapeutic value in
NMBA-treated rats with fully developed esophageal tumors. However, DFMO
appears to be an effective chemopreventive agent when administered in the diet
during the promotion / progression stages of NMBA-induced tumorigenesis.
Together, these findings illustrate the potential of certain chemopreventives to
produce diverse effects among animal model systems, and suggest the
importance of developing synergistic-based chemopreventives which inhibit
multiple processes during carcinogenesis.
Dedicated to my sisters, Nighat, Rukhsana, my brothers, Hameed and Tariq and to my parents, Dr. Khwaja and Azra Aziz
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TABLE OF CONTENTS

Abstract ............................................................................................................. ii
Dedication ......................................................................................................... iv
Acknowledgements ............................................................................................... v
Vita ..................................................................................................................... vii
List of Tables ...................................................................................................... xiii
List of Figures ....................................................................................................... xv

Chapters:

1. Introduction ...................................................................................................... 1
   1.1. Esophageal cancer in humans ................................................................. 1
       1.1.1. Overview of esophageal cancer epidemiology ......................... 1
       1.1.2. Risk factors associated with human esophageal SCC ............. 9
       1.1.3. Alcohol and tobacco use and human esophageal SCC .......... 11
       1.1.4. Nutritional and dietary factors and esophageal cancer ............ 17
       1.1.5. Other risk factors associated with esophageal SCC .............. 23
   1.2. The F-344 rat esophageal cancer model ............................................. 25
       1.2.1. N-nitrosoamines and rat esophageal tumorigenesis ............... 26
       1.2.2. Metabolism of NMBA ................................................................. 31
       1.2.3. Pathology and histopathology of NMBA-induced rat esophageal
tumorigenesis ................................................................................................. 36
       1.2.4. Molecular events associated with NMBA-induced rat esophageal
tumorigenesis ................................................................................................. 42
   1.3. Chemoprevention of esophageal cancer .............................................. 45
   1.4. Food components of esophageal cancer prevention ......................... 48
   1.5. Inhibitors of polyamine synthesis ......................................................... 51
   1.6. Proposed research ................................................................................. 58

2. The effect of freeze-dried blueberries on N-nitrosomethylbenzylamine induced
tumorigenesis ................................................................................................. 62
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Risk factors associated with the development of squamous cell carcinoma of the esophagus</td>
<td>10</td>
</tr>
<tr>
<td>1.2</td>
<td>Carcinogens in cigarette smoke and tars</td>
<td>15</td>
</tr>
<tr>
<td>1.3</td>
<td>N-nitrosamines carcinogenic for the rat esophagus</td>
<td>28</td>
</tr>
<tr>
<td>1.4</td>
<td>Classification and examples of cancer chemopreventive agents based on mechanism of action</td>
<td>50</td>
</tr>
<tr>
<td>1.5</td>
<td>Early known polyamines and their occurrence in cells</td>
<td>52</td>
</tr>
<tr>
<td>1.6</td>
<td>Components of different berry types used in chemoprevention studies</td>
<td>61</td>
</tr>
<tr>
<td>2.1</td>
<td>Experimental design for freeze-dried blueberry anti-initiation bioassay</td>
<td>72</td>
</tr>
<tr>
<td>2.2</td>
<td>Experimental design for DNA adduct study</td>
<td>72</td>
</tr>
<tr>
<td>2.3</td>
<td>Effect of freeze-dried blueberries on NMBA-induced tumorigenesis in the rat esophagus</td>
<td>76</td>
</tr>
<tr>
<td>2.4</td>
<td>Effect of freeze-dried blueberries on formation of O(^6)-methylguanine in the esophagus of rats treated with NMBA</td>
<td>76</td>
</tr>
<tr>
<td>3.1</td>
<td>Experimental design for the DFMO post-initiation bioassay</td>
<td>92</td>
</tr>
<tr>
<td>3.2</td>
<td>Effects of difluoromethylornithine on NMBA-induced preneoplastic esophageal lesions in the F344 rat</td>
<td>96</td>
</tr>
<tr>
<td>3.3</td>
<td>Anti-promotion/progression effects of difluoromethylornithine NMBA-induced esophageal tumorigenesis in the F344 rat</td>
<td>97</td>
</tr>
</tbody>
</table>
4.1 Experimental design of therapeutic bioassay using freeze-dried black raspberries ............................................................. 115

4.2 Effects of freeze-dried black raspberries on tumor development in N MBA-treated F344 rats (Week 26 of the study) ................................................................. 122

4.3 Descriptive statistics for tumor number by treatment group for black raspberry therapeutic study ................................................................. 125

4.4 Descriptive statistics for average tumor volume by treatment group for black raspberry therapeutic study ................................................................. 125

4.5 Kaplan-Meier survival estimates by group for black raspberry therapeutic study ................................................................. 125

4.6 Calculated probability of survival by group for black raspberry therapeutic study ................................................................. 126

4.7 Effects of freeze-dried black raspberries on PCNA labeling index in the F344 rat esophagus (Week 26 of the study) ................................................................. 126
LIST OF FIGURES

Table | Page
--- | ---
1.5 Pathways of NMBA metabolism | 32
1.6 Methylene hydroxylation pathway of \( N \)-nitrosomethylbenzylamine metabolic activation | 33
1.7 Histologic diagram of rat esophagus in longitudinal section | 37
1.8 Classification of microscopic histopathological and morphogenetic changes that occur during NMBA-induced esophageal tumorigenesis in the F344 rat esophagus | 40
1.5 Molecular events associated with NMBA-induced esophageal tumorigenesis | 43
1.6 Polyamine biosynthesis in mammalian tissues | 53
1.7 Chemical structure of DFMO | 54
2.1 Experimental protocol for the anti-initiation bioassay | 73
2.2 Experimental protocol for the DNA adduct study | 73
2.3 Body weight data for freeze-dried blueberries anti-initiation bioassay | 75
2.4 Food consumption data for freeze-dried blueberries anti-initiation bioassay | 75
3.1 Experimental protocol for the DFMO post-initiation bioassay | 93
3.2 Effect of DFMO on mean body weight in a post-initiation bioassay | 95
3.3 Effect of DFMO on food consumption in a post-initiation bioassay | 95
4.1 Experimental protocol for freeze-dried black raspberries therapeutic bioassay…………………………………………………………………………116
4.2 Graph showing body weights of rats after being fed freeze-dried black raspberries at week 19…………………………………………………………121
4.3 Esophageal tissue sections stained with PCNA……………………………………127
CHAPTER 1

INTRODUCTION

1.1 Esophageal cancer in humans

1.1.1 Overview of esophageal cancer epidemiology

Esophageal cancer ranks eighth in cancer incidence worldwide and is the fifth most common cause of cancer death (1-5). Current data indicate that approximately 13,000 deaths are expected from esophageal cancer in 2003, nearly matching the number of new cases (1-2, 6, 7). In terms of incidence and prevalence of the disease, developing regions of the world account for nearly 85% of all esophageal cancer cases (8, 9). Regions of the world that have the highest incidence rates include the area referred to as the “esophageal cancer belt”. This region includes countries located mostly in Central Asia such as China, Iran, and the former Soviet Union (10). Other countries with high occurrences of the disease include the Transkei region of South Africa, Afghanistan, Rhodesia, India, France and Puerto Rico (10-12). In terms of epidemiology at the global level, esophageal cancer rates are divided into three groups. In Group 1, cancer rates are high and include countries like Iran and China. Predominant causes for the disease are attributed to diet including
micronutrient deficiencies, low levels of protective factors in fruits and vegetables, and consumption of foods containing mytotoxins and high levels of carcinogens; i.e. nitrosamines (12, 13). In Group 2 countries, the cancer rate is relatively low and includes most of Europe and USA. Major causes of the disease are attributed to excessive tobacco and alcohol consumption accompanied by dietary deficiencies (12, 13). Group 3 countries such as Japan and South Africa have an intermediate cancer incidence (12, 13). Risk factors for Japan include consumption of hot tea gruel and toxic plants; i.e. braken while in South Africa, pipe smoking seems to be of more importance (10, 14).

Compared to the other countries mentioned, the highest incidence and mortality rates from this disease occur in the southern parts of the Taihang Mountains on the borders of Henan, Shansi, and Hebei provinces in China (15). Age-adjusted mortality rates for the Linxian region in Henan province were 151/100,000 and 115/100,000 for males and females, respectively (10, 16). Although the incidence in Europe is low, mortality rates for Switzerland, Scotland, Ireland, and England and Wales are high (17, 19). Other countries with high mortality rates include Singapore, Puerto Rico, and Chile (10). At the global level, geography of the region often is a major factor for the incidence of the disease (10). Conditions in climate, types of agriculture which determines types of food available, the occupations, life-styles, and socioeconomic status of the population play a role in the risk for the disease. High incidence regions exist in areas where there is a low consumption of fruits, vegetables, and animal food,
and where cereals form the staple diet. Rates can be high due to limited availability of fresh food, and this may occur especially in winter months (13).

Because of its wide variation in incidence rates, esophageal cancer epidemiology is very unusual. This is true both between and within individual countries. In France for example, the overall incidence is generally low despite the fact that, in the regions of Brittany and Normandy the incidence is high. Wide variations in incidence have been studied most often in China. High rates of 80 per 100,000 have been reported in the Taihang mountains in the Linxian region while rates of 20 per 100,000 are reported in neighboring county of Fanxian. Studies have ruled out economic factors for the uneven incidence of the disease in these areas (17).

Another interesting feature concerning esophageal cancer is the reporting of and changing occurrence of, the disease (6, 10, 20). In Northern Iran, esophageal cancer has been well recognized for over 800 years. This is not the case in South Africa where the disease was rare. In the last 12 years, however, the disease has become the most common cancer among black men in South Africa (10, 21, 22). This may have been the result of an increase in salary allowing an increase in the use of alcohol and tobacco. In the country of Zulus, no cases of esophageal cancer were reported prior to 1960. In the United States, the high rates reported among nonwhites also represent a recent increase in this population. The rates in whites however, have remained stable over time, except for adenocarcinoma which is increasing (7, 23-25).
A final interesting feature is the vast distribution of incidence rates among different parts of the world. In most areas of Europe and North America, the incidence rates are below 6 per 100,000 for men and 3 per 100,000 for women. There are regions in Iran and China in which the rates are over 100 per 100,000 for both sexes. Residents of Linxian, the geographic area which includes the provinces of Henan, Hebei, and Shanxi in the mainland of China, have incidence rates of around 30 to 50 per 100,000 and occasionally as high as 200 per 100,000. In addition, they have a 6-7% probability of dying from esophageal cancer (10, 20).

There is clear evidence of gender and racial differences in the development of esophageal SCC (12). Worldwide, males are two to three times more likely to develop the disease as females. There are a few notable exceptions. In the highest risk area of Iran, for example, annual incidence rates for females are 263 per 100,000 compared to 203 per 100,000 for males (12, 13). Another exception is in the case of women who have Plummer-Vinson syndrome, a syndrome associated with iron deficiency and occurs more often in women than men. Women tend to be more severely affected by poor diet compared to men. African Americans are more likely than Caucasians to develop the disease. In the U.S., the incidence rate for African Americans was 24 per 100,000 compared to 6.5 per 100,000 for Caucasians (10, 12, 26, 27). In fact, it is ranked as one of the five leading causes of cancer-related death in African-American men. Mortality rates of blacks compared to whites is also higher (27). In addition, African-Americans have poorer survival for the disease.
A combination of socioeconomic factors such as access to health care, financial costs, or other factors such as differences in alcohol and tobacco consumption may play a role for the variation in survival rates (10, 12). As with other cancers, there is an increased incidence and mortality for those over the age of 40 usually indicating cumulative effects of environmental agents.

Esophageal cancer is of two major types; squamous cell carcinoma (SCC) or epidermoid carcinoma which occurs principally in the middle and distal portion of the esophagus and, adenocarcinoma which arises at the distal portion of the esophagus. Approximately 90% of all esophageal cancers worldwide are squamous cell carcinomas (9, 10). The disease is less common in the United States. Adenocarcinomas, which arise from a lesion termed Barrett’s esophagus, account for about 5% of esophageal malignancies (9). Unlike SCC, adenocarcinoma of the esophagus has been rapidly emerging particularly in the United States and parts of Europe (10, 28). This increase may be associated with an increase in Barrett’s esophagus which will be described later. The other 5% of esophageal cancers are mesenchymal tumors or metastases from other sites (9, 16).

As indicated above, esophageal SCC accounts for 90% of all esophageal cancers worldwide (29). In the United States, however, esophageal SCC accounts for less than 50% of esophageal cancers, 1.5-2% of all cancers and approximately 5-7% of all gastrointestinal malignancies. Approximately 50% of SCC is found in the middle third of the esophagus, 30% are seen in the distal third of the esophagus, and 20% in the upper third. Esophageal SCC’s arise
from the basal epithelium of the esophagus. The tumors rapidly invade surrounding tissues and metastasize. Cells, in SCC, are polygonal, oval or spindle-shaped, and the tissue has a distinct stromal-epithelial interface. At the microscopic level, SCC can range from well-differentiated tumors with keratinization, moderate nuclear atypica and minimal necrosis to poorly differentiated tumors with a high mitotic index and large areas of necrosis (16, 30). Epithelial dysplasia, the principal precursor to esophageal SCC, is characterized by accumulation of atypical cells with nuclear hyperchromasia, abnormally clumped chromatin, and loss of polarity. Invasion by SCC occurs by intraesophageal spread, direct extension, and lymphatic or hematogenous metastasis. Studies have shown that SCC develops through a series of phases ranging from mild to severe dysplasia, then developing to carcinoma in situ and finally to invasive carcinoma (16). Squamous cell carcinomas are graded according to their degree of differentiation, and are characterized as either well, moderately, or poorly differentiated. Well-differentiated tumors exhibit an orderly stratification, noticeable cellular bridges, and keratin formation. In contrast, poorly differentiated tumors are noted for their lack of keratinization and lack of cellular bridges. Moderate carcinomas fall somewhat in between the two and exhibit features that overlap both types. Regional lymph node metastasis occurs in 42-67% of cases. Distant metastasis may be present in 25% to 30% of patients at the time of diagnosis, and in 50% of patients at the time of autopsy. The liver, lungs and bone are the most frequent sites of metastasis.
The prognosis for esophageal SCC is poor (16). This is especially true when the disease is diagnosed in the advanced metastatic stage. The five-year survival rate when the disease has metastasized is less than 10% (this is a significant increase compared to four decades ago; which was approximately 2%) (16, 27, 31). This low survival rate may be attributed to the fact that specific symptoms may not appear until the disease is well developed, thus limiting effective treatment options. The only symptoms that are evident are weight loss, difficulty in swallowing, vomiting, gastric irritation, and dyspnea. It is important to note, however, that dysphagia does not occur until at least 60% of the esophageal circumference is lined with cancer (20).

Adenocarcinomas, which arise from a preneoplastic lesion termed Barrett's esophagus, account for 5% of esophageal malignancies. Barrett's esophagus occurs when normal squamous epithelium of the esophagus is replaced by a glandular epithelium. Adenocarcinoma is the second most common type of esophageal cancer worldwide, and is the most common type in the U.S. and in Europe (32). In the mid-1970's, adenocarcinomas represented only 15% of all esophageal cancers in the U.S. At present however, approximately 50% of new cases of esophageal cancer in the U.S. are adenocarcinomas. Adenocarcinomas are derived from glandular tissue and the tumor cells form recognizable glandular structures. The glandular structures are derived from three sources: (1) superficial and deep glands of the esophagus, such as the mucous glands, (2) embryonic remnants of glandular epithelium, or (3) metaplastic glandular epithelium. Histologically, adenocarcinomas exhibit
small or large glandular patterns (32). The cells lining the glands are represented by variable degrees of cytoplasmic differentiation and they usually have an increased nuclear/cytoplasmic ratio. As mentioned previously, adenocarcinomas occur mainly in the lower third of the esophagus, near the gastroesophageal junction, and arise principally from a premalignant condition known as Barrett’s esophagus (27). Studies show that 59-86% of adenocarcinomas arise from Barrett’s esophagus. Barrett’s esophagus is a condition in which metaplastic columnar epithelium replaces the distal squamous mucosa due to prolonged exposure of the esophageal mucosa to stomach acid from gastroesophageal reflux. This condition arises when the stomach contents “back up” or reflux into the esophagus irritating the lower esophagus. Recurrent long-term reflux results in continued inflammation and ulceration of the squamous mucosa. Patients are 30-40 times more likely to develop adenocarcinoma from Barrett’s esophagus than from non-Barrett’s epithelium. Thus, the increase in Barrett’s esophagus could explain why adenocarcinomas are on the rise (33). Risk factors associated with adenocarcinomas include high intake of red meat and polyunsaturated fats, low consumption of fruits and vegetables, fish, vitamins, and dietary fiber, obesity, cigarette smoking and alcohol consumption and medications that relax the esophageal sphincter and cause immunosuppression (12, 34). Other factors that could account for the rise in adenocarcinomas include more accurate diagnosis of esophageal cancer, *Helibactor pylori* infection, and duodenalgastric reflux.
As stated earlier, adenocarcinomas have been steadily on the rise in the United States and occur less frequently in Middle and far Eastern areas of the world. As with SCC, incidence and mortality patterns are higher among males compared to females (26, 35). Unlike SCC, however, adenocarcinoma of the esophagus rates among Caucasians is higher compared to African Americans (26, 28, 36).

1.1.2. Risk factors associated with human esophageal SCC

There is no single factor that can account for the pattern of esophageal SCC in high-risk areas. Rather, the disease appears to be caused by the combination of multiple factors. Risk factors associated with the development of esophageal SCC are listed in Table 1.1. They can be divided into a number of categories including lifestyle, dietary, environmental and occupational factors, nutritional deficiencies, exposure to infectious agents and radiation therapy, chronic irritation of the esophagus, genetic predisposition, and other associated diseases (9, 16, 29, 37). These risk factors work in different ways. Some appear to act directly on initiation events in tumor development while others act later in the promotion / progression stages of cancer. It should be noted that the development of esophageal SCC may almost certainly be the result of a combination between one or all of these risk factors. Epidemiological studies have indicated that variation among incidence rates correlate with certain risk factors. In China and South Africa, for example, extensive research has suggested that \( N \)-nitroso compounds, including \( N \)-nitrosomethylbenzylamine (NMBA), and their precursors are probable etiological factors for esophageal
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<thead>
<tr>
<th>Risk Factors</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol, dietary factors, tobacco</td>
<td>Nitrosamines (pickled vegetables, daily barbecue, highly salted foods,</td>
</tr>
<tr>
<td>smoke</td>
<td>etc.)</td>
</tr>
<tr>
<td></td>
<td>Mycotoxins (<em>Fusarium</em>, <em>Alternaria</em>, <em>Geotrichum</em>, <em>Aspergillus</em>)</td>
</tr>
<tr>
<td></td>
<td>Corn consumption</td>
</tr>
<tr>
<td></td>
<td>Retinol</td>
</tr>
<tr>
<td>Nutritional deficiencies</td>
<td>Vitamins A, C, E, niacin, riboflavin, zinc</td>
</tr>
<tr>
<td></td>
<td>Low intake of fresh fruit and vegetables</td>
</tr>
<tr>
<td>Environmental factors</td>
<td>Low socioeconomic status</td>
</tr>
<tr>
<td></td>
<td>Soil deficiencies (molybdenum, manganese, and zinc)</td>
</tr>
<tr>
<td>Occupational factors</td>
<td>Vulcanization workers</td>
</tr>
<tr>
<td></td>
<td>Plumbers and pipefitters</td>
</tr>
<tr>
<td></td>
<td>Bartenders and brewery workers</td>
</tr>
<tr>
<td>Infectious agents</td>
<td>Papillomavirus, fungi and bacteria</td>
</tr>
<tr>
<td>Chronic irritation</td>
<td>Thermal and mechanical (spicy and hot foods, mate drinking, rapid eating)</td>
</tr>
<tr>
<td></td>
<td>Achalasia</td>
</tr>
<tr>
<td></td>
<td>Chronic lye irritation</td>
</tr>
<tr>
<td></td>
<td>Esophageal diverticulum</td>
</tr>
<tr>
<td>Radiation therapy</td>
<td></td>
</tr>
<tr>
<td>Injection sclerotherapy</td>
<td></td>
</tr>
<tr>
<td>Genetic predisposition</td>
<td></td>
</tr>
<tr>
<td>Other tumors of the upper digestive tract</td>
<td></td>
</tr>
<tr>
<td>Gastric resection</td>
<td></td>
</tr>
<tr>
<td>Associated diseases</td>
<td>Plummer-Vinson syndrome</td>
</tr>
<tr>
<td></td>
<td>Celiac disease</td>
</tr>
<tr>
<td></td>
<td>Tylosis</td>
</tr>
<tr>
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<td>Scleroderma</td>
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Table 1.1: Risk factors for squamous cell carcinoma of the esophagus. Modified from Ribeiro *et. al*, 1996 (38)
SCC in the high incidence areas (17). In other areas of China, consumption of salt-pickled, salt-cured and moldy foods (some of these products are frequently contaminated with N-nitrosamine carcinogens and/or fungal toxins) are also implicated in the pathogenesis of the disease.

In parts of India and Pakistan, consumption of hot, spicy foods and beverages with meals could explain chronic esophageal irritation leading to the development of the disease. While in many parts of Europe, synergistic effects using tobacco and alcohol are associated with the pathogenesis of the disease (9, 13, 16, 39).

1.1.3. Alcohol and tobacco use and human esophageal cancer

The two most significant risk factors for esophageal SCC in the Western world are cigarette smoking and alcohol consumption. While tobacco smoking by itself places one at higher risk of the disease, it is suggested that smoking and heavy alcohol use act in a synergistic manner in increasing the risk for the disease. Large prospective cohort studies have consistently reported an association between smoking, tobacco use and the risk for SCC. In fact, both of these factors account for over 90% of the risk associated with esophageal cancer. When combined, these two factors increase the risk from 25 to 100-fold when compared to either factor alone. Studies from Hirayama estimated that persons in Japan, who both drank daily and smoked, had a rate of esophageal cancer 3 times higher than normal (17). The risk was lowest for those who drank beer and increased with saki, whisky, and shochu consumption. Research cited
by Wynder and Bross suggest that an 80% reduction in incidence in the United States would occur in the absence of smoking and heavy drinking (37).

In terms of alcohol consumption, the risk of developing esophageal cancer in the United States is increased in heavy drinkers (10-fold for beer and 25-fold for whisky) compared to smoking-matched nondrinkers (17). This trend is also observed at the global level. Studies by Hirayama estimated that in Normandy and Brittany, which produce a large quantity of spirits and distillates, the esophageal tumor incidence is high due to alcohol use (17). Epidemiological studies in Northeast France show strong evidence of esophageal cancer and excess consumption of apple cider and its distillates. Higher rates in France could also be attributed to an increased consumption of red wines which contain high amounts of tannins. This is especially true for red Bordeaux that has been aged 2-3 years in barrels. In Northern Italy, the wine is left standing in contact with seeds and grapes in order to increase tannin content. This is not the case in the U.S.A. which has adopted procedures to reduce tannin content of wine (13). Cook et al. showed a strong correlation between maize-manufactured beer and the geographic distribution of esophageal cancer in Africa (17). Specifically, he found that areas such as the Transkei region of South Africa and western Kenya where corn production is heavily used in producing beer had higher incidences of esophageal carcinoma compared to Uganda and West Africa where little or no maize beer is produced. Further studies by Marasas et. al reported these maize kernels grown in the high incidence regions of South Africa are contaminated with the fungus, *Fusarium* (17). Westhuizen et. al have found that certain
regions of Brazil contain corn high in *Fusarium* and *Fumonisin* that contaminates corn (40). There is evidence that the types of alcohol consumed affect the incidence rates. For example, risk among those who consume whisky and distillates is usually twice that from beer drinkers. Higher incidences are seen among beer than wine drinkers. The length of time that one consumes alcohol has not been reported as a risk factor for esophageal SCC in smokers. However, it is likely to be important. An interesting observation, however, has been seen in South America. Studies have reported that former alcohol drinkers remain at a higher risk for esophageal SCC than non-drinkers for a period of 19 years (41). High incidence has been associated with certain occupations where high alcohol exposure is present such as bartenders, brewery employees, waiters, and hotel keepers (37, 38).

Although heavy alcohol use is an important risk factor in the causation of SCC in Westernized countries, it seems to have little or no role in some of the major incidence regions. Moslems from areas of Northeastern Iran, India, Pakistan, and the former Soviet Union, for example, have higher incidences despite the fact that alcohol use is forbidden due to religious guidelines (the high incidence may be associated with opium, tobacco use, hot tea intake or the combination of all as discussed below) (17). In the Linxian region of China, another high incidence area, little or no alcohol is consumed yet Linxian is one of the areas with the highest incidence of the disease [an exception to this are fisherman who reside on the Nan’ao Island of Guangdong province] (9, 20).
Although the exact mechanism of alcohol consumption and cancer risk is not entirely known, ethanol serves as a solvent for tobacco carcinogens, and toxins, thus increasing exposure of these agents to reach the epithelial cell in the esophagus. Ethanol also influences cancer risk in other ways such as its stimulatory effect on the metabolic activation of carcinogens and inhibitory effect on carcinogen detoxification. In addition, ethanol has the potential at increasing cellular exposure to various oxidants resulting in an increased risk of DNA damage and neoplastic transformation. The toxic agents contained in ethanol including ketones, aldehydes, and phenols can also produce toxic effects to the esophagus once consumed. And finally, individuals who consume ethanol tend to be nutritionally deficient and/or have diets that lack many essential vitamins and minerals and as a result, they have insufficient intake of chemopreventive agents from the diet (29).

Extensive use of tobacco and tobacco related products increases the risk for cancer of lung, kidney, bladder, oropharynx, larynx, pancreas, brain, and certain leukemias (42). This is the case not only for cigarette smokers but also for pipe and cigar smokers and for chewers of tobacco and betel quid. As mentioned earlier, tobacco products and cigarette smoke contain a large number of chemical carcinogens and toxins such as nitrosamines, polycyclic aromatic hydrocarbons, aromatic amines, epoxides, lactones, peroxo compounds, halo-esters and various aldehydes and phenols (Table 1.2). The most suggestive evidence for the role of tobacco as a carcinogen for the esophagus comes from Auerbach et al. (17). He reported that among American men, 6.6% of those who
### Aromatic hydrocarbons

- Benzo[a]pyrene
- Benzo[a]anthracene
- Benzo[b]fluoranthene
- Benzo[j]fluoranthene
- Chrysene
- Dibenzo[a,h]acridine

### Nitroso compounds

- N-nitrosodimethylamine
- N-nitrosodiethylamine
- N-nitrosoethylmethylamine
- N-nitrosodiethanolamine
- N-nitrosodi-n-butylamine
- 4-(methylnitrosoamino-1-(3-pyridyl)-1-butanone
- N-nitrosoanatabine

### Epoxides, lactones, peroxo compounds, and halo-ethers

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<td>1,2,6,7-Diepoxyheptane</td>
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<td>1,2,4,5-Diepoxybutane</td>
<td>1,2,4,5-Diepoxybutane</td>
</tr>
<tr>
<td>Vinylcyclohexane hydroperoxide</td>
<td>Bis(chloromethyl)ether</td>
</tr>
<tr>
<td>Glyceraldehyde</td>
<td>Glyceraldehyde</td>
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Table 1.2: Carcinogens in cigarette smoke and tars. From Hoffmann and Hecht; Brunnemann and Hoffmann; Van Duuren; McCormick et al. (29)
did not smoke showed signs of atypical nuclei in the basal layer and none of them had carcinoma \textit{in situ}. In the smokers, however, 79.8\% of cases had atypical nuclei while carcinoma \textit{in situ} was found in the epithelium of 1.9\% of cases. Furthermore, the epithelium tended to be thicker for smokers at the time of death compared to nonsmokers thus indicating a hyperplastic response (9). In terms of defining the mechanism of tobacco, it is suspected that tobacco may act as an initiating agent while ethanol may serve as a promoter. Many of the toxic and carcinogenic agents found in tobacco such as benzo(a)pyrene, and the tobacco-specific nitrosamines, \textit{4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)} and \textit{N’-nitrosonornicotine (NNN)} have the potential of producing adverse effects in the esophagus.

The chewing of tobacco, betel, or a combination of the two with lime may explain the relatively high occurrence of esophageal and oral cavity cancer in regions of the world like Ceylon, Pakistan, India, Burma, and other Southeastern Asian countries. Stoner and Rustgi reported that chronic ingestion of these products over time cause the mucosa to become irritated thus eventually damaging the epithelium (9). This in turn results in hyperplastic and neoplastic changes of the epithelial cells (29). Clinical studies of patients with esophageal SCC suggest that opium and the combustion products that are released from opium are risk factors for the disease. This may explain the surprisingly high incidence in certain high risk areas of northeast Iran and the Transki region of Africa. Crude opium itself does not exhibit any mutagenic activity. However, the pyrolysis and condensates produced when opium is smoked are more mutagenic
and carcinogenic than tobacco smoke condensates (17). These pyrolyzed substances display mutagenic activity in *Salmonella typhimurium* strains TA<sub>98</sub> and TA<sub>100</sub> in rat liver microsomes (13). A high incidence of esophageal cancer occurs among Iranians who use opium (as evidenced by the presence of a morphine metabolite in their urine) from those which did not use opium (13).

1.1.4. **Nutritional and dietary factors and human esophageal cancer**

Other risk factors that may contribute to esophageal SCC include a diet low in beta-carotene, vitamins A, C, and B, magnesium, zinc, riboflavin, niacin, and selenium, and fruits and vegetables. Contaminants in food and water also appear to play a role in increasing risk. Combinations of one or more of these risk factors are evident in many high risk areas. Frequent consumption of hot beverages, such as tea, may weaken the esophageal lining over time and increase risk. Ingestion of highly salted foods and of pickled vegetables also contributes to the risk. These foods contain *N*-nitrosamine compounds and several fungal toxins which are potent carcinogens. Fungal toxins may contribute to growing irritation and infection in the esophagus. The presence of infection may weaken the esophagus over time thus increasing the risk.

Contaminants present in food and water that have been implicated as risk factors for esophageal SCC include nitrates, nitrites and secondary and tertiary amines which are precursors for nitrosamines *in vivo*. These substances serve as precursors for nitrosamines in the stomach. Enzymes in the stomach, under acidic conditions, have the ability to form *N*-nitrosamines and other *N*-nitroso compounds from the reaction of nitrites and amines. In addition to precursors for
N-nitroso compounds, some foods such as rice and wheat are contaminated with a number of fungal species such *Fusarium, Alternaria, Geotrichium, Aspergillus, Cladosporium,* and *Penicillium* which produce mycotoxins that are both mutagenic and carcinogenic once ingested. This is especially true in the diet in Linxian, China, and Transkei region of South Africa where fungal contamination of grains is common. Fungi once ingested, can also invade esophageal tissue leading to local inflammation, epithelial hyperplasia, dysplasia and other neoplastic changes associated with the esophagus. Fungal infection, especially with *Candida albicans,* has been positively associated with hyperplastic growth of esophageal epithelium (9). Further studies showed that common species of fungus such as *Fusarium, Geotrichium, Aspergillus,* and other genera could not only reduce nitrates to nitrites, but also decompose proteins thus increasing the amount of amines in foods, leading to a higher amount of nitrosamine formation (9).

Salt-cured, salt-pickled and moldy foods, which are frequently consumed in high incidence regions, also contain fungal toxins and nitrosamines. Specifically, trace amounts of nitrosamines such as NDEA, NDMA, NMBA, and *N-(1-methylacetonyl-N-3-methylbutyl)* nitrosamine have been identified in the cornbread and pickled vegetables consumed in high incidence areas of China and South Africa. In fact, chronic esophagitis is common among younger people of Huixian where consumption of pickled vegetables starts during adolescence. Since the nitrosamines contained in these foods are low (usually less than 10 parts per billion) and not harmful at this concentration, their precursors,
specifically nitrates, nitrites, and secondary amines have the ability to produce $N$-nitroso compounds in the stomach under acidic conditions (discussed above). Consumption of smoked fish, salted tea, and sun-dried foodstuffs may contribute to the relatively high incidence in the province of Kashmir in India. Drying raw foodstuffs under the open sun provides high concentrations of $N$-nitroso compounds. Studies from India indicate that the food additive kalakhar, a highly alkaline substance made from charring bananas, plays a significant role in the etiology of esophageal SCC in India (39). $N$-nitroso components found in water may be another consumable risk factor for esophageal SCC. Among Saudi Arabians, for example, petroleum oil impurities in drinking water appear to increase risk. This was the case in Gassim, Saudia Arabia, where five out of six samples contained increased levels of petroleum oil. Chemicals in petroleum are highly carcinogenic and include polyaromatic hydrocarbons, benzenes and lower alkylbenzenes (17). In the Henan province of China, epidemiological studies revealed high concentrations of nitrates and nitrites, ranging from 0 to 2.63 mg/liter, within the drinking water of peasants (17, 43).

There is some evidence suggesting a role for hot tea and beverage consumption in the etiology of esophageal SCC. This is especially true in high risk populations where the beverage is often drunk while “burning hot”. Consumption of hot beverages tends to damage the mucosa and, over time, weakens the mucosal epithelial lining of the esophagus. This “thermal injury” to the esophagus increases the reactivity of fungal species, tannins, etc. thus further weakening the lining. High incidence areas such as in China,
northeastern Iran, Puerto Rico, Chaoshan region in Guangdong province and other regions where the drinking of hot beverages, especially tea, is a common practice have higher rates of the disease. Studies in Japan, the former Soviet Union, and Northern Iran indicate that inhabitants from high-risk areas drink larger quantities of hot tea than those from low-risk areas (20). In Japan, the consumption of chagayu, rice gruel with hot tea, correlates positively with mortality from esophageal SCC. Chronic thermal irritation, high levels of tannin in the heated state, and the use of iron pots in preparation of chagayu may play a role in carcinogenesis. Braken, also widely consumed in Japan, also has been shown to increase risk especially when taken in combination with chagayu. Interestingly, studies done in Northeast Argentina, Southern Brazil, and Uruguay showed that mate drinkers (individuals who consume hot drinks taken through a metal tube that bring heat infusion to the esophagus) were 2.2 times more likely to develop histologically confirmed esophagitis than nonmate drinkers (44, 45). Further work to evaluate the importance of mate drinking and esophageal cancer risk is being done in the country of Paraguay where mate is consumed cold. In addition to evidence supporting hot tea and beverage consumption, hot temperature of food items, in combination with hot beverages, may also increase thermal injury to the esophageal mucosa thus weakening the esophageal tissue (46). This is especially true in areas of the world like India and Pakistan where hot food and beverages are consumed together (46, 47, 48).

Epidemiological studies suggest that a number of vitamins and trace mineral deficiencies are associated with an increased risk for esophageal SCC.
Deficiencies in vitamins A, C, E, B12, and riboflavin increase the risk. Vitamin A in plasma is lower in patients with esophageal SCC compared to those with nonsquamous cancer (49). Diets rich in beta-carotene may lower esophageal cancer risk. Vitamin A and its precursor, beta-carotene, have been shown to exhibit a variety of beneficial effects such as maintenance of normal cell proliferation and differentiation, cell-to-cell communication, and antioxidant activity. Fruits and vegetables containing vitamin C suggest that it protects against the disease. This theory that vitamin C is protective was challenged when blood ascorbate levels were not significantly different among Chinese with a high or low risk for esophageal cancer. Both vitamins C and E possess antioxidant activity and have been shown to inhibit the formation of nitrosamines from precursors (50). Folic acid and riboflavin are also deficient in individuals with the disease. Studies by Jankowski et al. found lower levels of folic acid and vitamins A, E, and B12 in the plasma of those with esophageal cellular dysplasia or carcinoma compared to normal patients (51). A study by Zheng et al. discovered that peasants with the disease in Linxian, China excreted 1000 µg less riboflavin in 4-hour urine samples than individuals who were disease-free (43). A decreased consumption of certain minerals such as zinc, selenium, molybdenum and other trace elements in crops, soil, and food has been found in patients with esophageal cancer (52). This is especially true in high risk areas of China and Iran where vitamin and mineral deficiencies are common, especially among families with low socioeconomic status (53). High levels of zinc exist in more expensive foods such as meat, fish and green and leafy vegetables.
Studies by Nomura et al. observed low levels of zinc in the hair, serum and in tumors of patients with esophageal carcinoma (54). The importance of dietary zinc has also been reported in animal studies since the esophagus is the most severely affected organ by zinc deficiency. Zinc-deficient rats had more esophageal tumors when administered \( N \)-nitrosomethylbenzylamine than normal rats (55). It has been speculated that lack of zinc increases microsomal activation of NMBA, thus leading to a higher production of the \( O^6 \)-methylguanine DNA adduct. In addition, other studies have demonstrated that zinc plays an important role in protecting against oxygen-free radical damage (50). Riboflavin deficiency, similar to zinc deficiency, tends to occur in poor rural communities especially among individuals with low socioeconomic status (17). As stated before, early evidence linking lack of riboflavin and iron with esophageal SCC occurred in studies of individuals with Plummer-Vinson syndrome. Plummer-Vinson syndrome is associated with riboflavin and iron (possibly some other minerals) deficiency. Women with Plummer-Vinson syndrome in Sweden, Norway, Scotland and Wales displayed symptoms of anemia and later developed esophageal cancer (56, 57). Decreased prevalence rates in these women were observed when riboflavin was added to the bread of the diet (57). Animal studies indicate that riboflavin deficiency can promote congenital malformations resulting in complete absence of the esophagus (13). Enzyme catalysts formed from riboflavin such as, flavine adenine dinucleotide (FAD) and flavomononucleotide (FMN), are essential for the activity of many oxidases and dehydrogenases (52, 54, 58, 59). These enzymes may play a role for maintaining and protecting
esophageal epithelial cells. Breakdown of these cells, as the result of riboflavin deficiency, could predispose the epithelium to carcinogens. In addition, Yang and Chen reported that flavo enzymes, enzymes formed from riboflavin, are important mixed-function oxidases aiding in the detoxification and activation of many carcinogens (11, 32).

Analyses performed in Linxian and Henan Province of China showed low concentrations of molybdenum in food (17, 53, 60). Although not extensively studied, molybdenum is an important constituent for the activity of certain enzymes such as nitrate reductase and various oxidases. Lack of molybdenum in the diet could lead to an accumulation of nitrates thus stimulating nitrosamine production in the stomach (13). In spite of these findings, a deficiency in any of these minerals does not appear to be a sufficient cause by itself for the development of esophageal SCC (discussed above).

1.1.5. Other risk factors associated with human esophageal cancer

Recent literature has cited the role of human papilloma virus (HPV) as another risk factor for esophageal SCC (9, 11, 61). The HPV genome is broken down into three regions which contain different regulatory genes. The early region contains the E1-E7 genes, the late region the L1-L2 genes, and the untranslated region contains unknown genes (9, 11, 61). The E6 gene product is involved in deregulating p53 while the E7 gene product inactivates the retinoblastoma protein. Deregulation and/or inactivation can lead to altered expression of these tumor suppressor genes. Other studies have found that various HPV genotypes associated with a high risk of other human cancers, such
as HPV-16, 18, and 33 are also positively associated with esophageal squamous cell carcinoma (11, 61). Recently, highly sensitive molecular techniques have been able to identify HPV-16 and HPV-18 in 15% of esophageal tumor samples while 10% of tumors contain the HPV genotype (29). Although still under investigation, the exact role of HPV contributing to carcinogenesis has not been determined.

Environmental exposures that contribute to SCC of the esophagus include high level exposure to asbestos, ionizing radiation and the ingestion of dust from animal hides (62). High-risk diseases, in addition to Plummer-Vinson syndrome, that predispose to esophageal SCC include achalasia, strictures associated with lye ingestion, and tylosis (38).

The combination of tobacco use, alcohol consumption, vitamin deficiency, and other risk factors facilitate esophageal carcinogenesis, indicating that this disease has a multifactorial etiology. In view of these exposures, one approach to the prevention of esophageal SCC is through changes in lifestyle, including avoidance of tobacco and alcohol. Additional benefits may be realized with the elimination of high salt foods that may be contaminated with microbial toxins, nitrosamines and their precursors. Chemoprevention, to address factors associated with the etiology and progression of the disease, is another viable approach. By using a rat model for esophageal SCC in which tumors are induced with the nitrosamine, NMBA, our laboratory and others have identified multiple chemopreventive agents for the esophagus (9, 16, 63-76). Moreover, clinical trials have identified some chemopreventive agents that appear to be
useful for reducing the risk for development of esophageal SCC in humans (9, 77-79).

1.2. The F-344 rat esophageal cancer model

The rat has been used most extensively as a model of human esophageal cancer. The reason for this is that tumors can be induced in this model system in a relatively short amount of time after a carcinogen is administered. Tumors can easily be induced by treatment of the rats with several nitrosamines, including the food contaminant, \(N\)-nitrosomethylbenzylamine (NMBA) and the tobacco-specific nitrosamine, \(N\)-nitrosonornicotine (NNN). Depending upon the dosing regimen, NMBA induces esophageal tumors in rats within 15 weeks or less with a 100% tumor incidence and multiple tumors per esophagus by 20 weeks (64-75). NNN, a cyclic nitrosamine, induces on average 1-2 tumors / esophagus in approximately 2 years (16) of continuous administration in the drinking water. Although one of the most potent lung carcinogens, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) does not induce tumors in the rat esophagus (29). Of all the nitrosamines isolated, NMBA appears to be one of the most selective carcinogens inducing tumors only in specific tissues such as the esophagus and nasal cavity of rats. Other species of animals such as hamsters, mice and guinea pigs are much less susceptible to NMBA-induced esophageal tumorigenesis (29). In addition, administration of \(N\)-nitrosoamines in this model system leads to progressive histopathological preneoplastic changes, such as simple leukoplakia, and dysplasia, similar to that observed in humans (16, 55, 80). Furthermore, this animal model also develops measurable levels of \(O^6\)-
methylguanine adducts once exposed to nitrosamines (16). Thus, the rat model is useful not only for detecting tumors but also for examining the metabolism and detoxification of NMBA.

1.2.1. N-nitrosamines and rat esophageal tumorigenesis

Nitrosamines play an important role in carcinogenesis (81, 82). Many nitrosamines are abundant in the environment, being present in food, drink, water, and widely used in industrial products. As stated earlier, an interesting feature about N-nitroso compounds is that they can be formed in the stomach from nitrites and amines (81). The carcinogenicity of nitrosamines may be enhanced by secondary risk factors such as consumption of alcoholic beverages, dietary deficiencies, or exposure to mycotoxins. More than 300 N-nitrosamines have been tested for carcinogenicity and half are of these are carcinogenic for the rat (82). Of all the N-nitroso compounds that have been found to be carcinogenic for the rat, NMBA is the most potent esophageal carcinogen. Some N-nitrosamines are highly potent esophageal carcinogens in animals whether given orally, or by the intraperitoneal, intravenous or subcutaneous routes.

There are two main classes of N-nitroso compounds, the chemically inert and chemically reactive (82). The chemically inert compounds are the N-nitrosamines which include NMBA. They require metabolic activation to forms that bind to DNA and produce cancer. The organ in which nitrosamines induce cancer is largely dependent on their ability to metabolize these compounds rather than their route of administration. In general, N-nitrosamines are not metabolized by either the brain or fetus. The chemically reactive nitroso compounds do not
require metabolic activation to induce cancer. These include the \( N \)-nitrosamides, \( N \)-nitrosoureas, \( N \)-nitrosoguanidines and the \( N \)-nitrosourethanes. The site of action of these compounds depends mainly on the route of administration and on which organ is exposed to the highest dose.

For most \( N \)-nitroso compounds, unless given orally, the esophagus is not a major target organ for carcinogenicity. \( N \)-nitrosamines can be either dialkyl compounds, symmetric or asymmetric, cyclic, aromatic, or heterocyclic (13) Table 1.3. Although some nitrosamines are esophageal carcinogens in the rat, many of them produce cancer in other organs, depending upon the animal species. In the hamster for example, only \( N \)-nitroso compounds which do not require metabolic activation produce cancer in the esophagus, while \( N \)-nitrosamines are inactive (82). \( N \)-nitroso-\( N \)-diethylamine (NDEA), a potent carcinogen for the liver and lung does not induce esophageal tumors when fed to different animal species, at different doses and over an extended period of time. In addition, minor changes in structure have been shown to have a dramatic effect on the target organ. NDEA is a potent carcinogen while \( N \)-nitroso-\( N \)-dimethylamine (NDMA) did not produce any esophageal tumors when fed to different animal species over an extended period of time. Symmetry appears to play a role in nitrosamine reactivity. Asymmetric nitrosamines are more reactive in the esophagus than symmetric nitrosamines. Among asymmetric nitrosamines, highly lipophilic ones tend to be more carcinogenic than those which are less lipophilic. Studies have shown that rat esophagus is the principal target organ for many asymmetrical nitrosamines (82). All the carcinogenic
<table>
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<td>(2-hydroxypropyl)amine</td>
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Table 1.3: N-nitrosamines carcinogenic for rat esophagus. Modified from Craddock, 1993 (13).
nitrosamines were also shown possess mutagenicity when they were incubated with *Salmonella typhimurium*.

Our laboratory has commonly used two protocols (a) a complete carinogenesis or anti-initiation bioassay and (b) a post-initiation or anti-promotion/progression bioassay to evaluate the efficacy of chemopreventive agents in the rat esophagus (16). In the first bioassay, the putative chemopreventive is given before during, and after administration of NMBA. In the second bioassay, the chemopreventive is given only after NMBA treatment is terminated (typically by the end of the fifth week). The complete carcinogenesis bioassay is effective for initial evaluation of chemopreventive agents since it is given during both the initiation and promotion/progression stages of tumor development while the post-initiation bioassay is used to determine the ability of the chemopreventive agents to inhibit the progression of preneoplastic lesions into papillomas. Studies by Siglin et. al investigated various short-term treatment regimens with NMBA in rat esophagus with the aim of developing a protocol for esophageal tumorigenesis that could subsequently be used in future studies (83). They concluded that abbreviated doses of NMBA at 7.5 or 10.0 mg/kg/body weight given over the course of 1-2 weeks led to excessive toxicity and resulted in animal death. Cumulative doses of NMBA administered at the same dose and time interval did not produce any tumors by 30 weeks. A cumulative dose of 7.5 mg/kg/body weight administered 3 times a week over the course of 5 weeks, however, did not produce any lethal toxicity in rats and resulted in tumor formation in the esophagus. Specifically, a cumulative dose of 7.5 mg/kg body
weight over 5 weeks led to a tumor incidence of 40% at week 10, 100% at week 20, and 100% at week 30, respectively, and yielded an average of two to three tumors per animal when evaluated at week 25. Based on these studies, it was concluded that administration of NMBA over a course of 5 weeks would be the most effective at producing tumors in the rat esophagus. Because cells are initiated at the end of 5 weeks, this dosing regimen is ideal for identifying suppressing agents in an anti-promotion/progression bioassay.

Administration of NMBA at a total dose of 7.5 mg/kg body weight over the course of 15 weeks will also induce esophageal tumors (83). In this protocol, NMBA is administered weekly at 0.5 mg/kg body weight leading to 100% incidence and multiplicity of approximately 10 tumors / rat at the end of 25 weeks. The reason for the higher tumor response when fractionating the dose over 15 weeks is unclear but may be due to a “promotional” effect of the NMBA. This promotional effect from the carcinogen makes this dosing regimen ideal for identifying agents that inhibit tumor initiation. Regardless of the dosing regimen used, a number of preneoplastic lesions are produced in the esophagus as the result of multiple treatments with NMBA. These include simple hyperplasia, epithelial dysplasia (simple hyperplasia and epithelial dysplasia can be broken down into stages of mild, moderate, and severe), squamous cell papillomas, and occasional squamous cell carcinomas. The multistage pattern closely mimics what would be observed in humans diagnosed with SCC. Due to animal respiratory distress, few if any NMBA-treated animals are able to survive long enough to develop carcinomas.
1.2.2. Metabolism of NMBA

As mentioned in the previous section, NMBA falls into a class of carcinogens called nitrosamines. Nitrosamines are found in tobacco smoke, cooked or broiled foods, and in certain chemical mixtures (81). They are organotropic carcinogens in experimental animals, and tumor development in specific organs is highly dependent on both the nitrosamine and animal species (82). NMBA is a potent and specific carcinogen which induces papillomas and carcinomas in the rat esophagus. NMBA is an asymmetric procancerogen and thus, must be metabolically activated to induce its carcinogenic effect. NMBA is metabolized through a series of steps to form a carbonium ion which binds to guanine in DNA and produces mutations leading to cancer (Figure 1.1). The first step in NMBA metabolism is alpha-hydroxylation of the carbon adjacent to the benzene ring. This step is catalyzed by CYP450 mixed function oxidases. Different P450 enzymes have broad overlapping specificities, each metabolizing a range of nitrosamines. In the rat esophagus, CYP2A3 appears to be important for catalyzing this reaction while in the human esophagus, the reaction is catalyzed principally by CYP2E1 (84). Since NMBA is asymmetric, it has the capacity to be metabolized by two potential pathways. The first pathway (methylene pathway – Figure 1.2) results in the formation of a methylating agent through alpha hydroxylation of the carbon closest to the benzene ring. This results in the formation of an alpha-hydroxynitrosamine which is then converted to the electrophile, methylazo hydroxide and benzaldehyde. Benzaldehyde, which is spontaneously released, may be converted to benzoic
Figure 1.1: Pathways of NMBA metabolism.
Figure 1.2: Primary methylene hydroxylation pathway of NMBA activation
acid and benzyl alcohol. The unstable methyldiazohydroxide loses a hydroxyl group and can then is converted to an unstable methyldiazonium ion which loses its nitroso group leaving a positively charged carbonium ion. The carbonium ion is an excellent electrophile which has the ability to attack the nitrogen and oxygen atoms of purine and pyrimidine bases in DNA resulting in the formation of adducts at specific sites [twelve have been noted (85)]. Specifically, the carbonium ion attaches itself to the O\(^6\) and/or N-7 positions of guanine. The major alkylated base to be formed is 7-methylguanine (7-MeGua) and the 6-methylguanine (O\(^6\)-MeGua) adducts. The initial ratio of 7-MeGua to O\(^6\) MeGua is 10:1. Another DNA adduct, 4-alkylthymidine, is also formed by nitrosamine metabolism (none have been reported using NMBA to date) however, its importance in carcinogenesis is limited. The 7-MeGua adduct occurs more frequently than the O\(^6\) MeGua adduct. Unlike the O\(^6\) MeGua adduct, the 7-MeGua adduct is lost from DNA by slow non-enzymic depurination and has the ability to be repaired slowly by certain enzymes found in the liver. The O\(^6\) MeGua adduct also has the ability to repair itself by the enzyme O\(^6\)-alkylguanine-DNA-alkyl transferase. This reaction transfers the methyl group in guanine to a sulfhydyl group in the cysteine residue within the repair protein. The rate of repair is determined by the amount of repair protein in the cell nucleus. The amount of protein varies depending on the organ. In the rat, the liver contains the highest concentration while the kidney, esophagus, and brain have the lowest amount (82). The esophagus further loses its repairing ability after subsequent treatment of NMBA. Several days may be needed for the protein to
be restored. Thus, it is the O\textsuperscript{6} MeGua adduct that is more likely to be implicated in carcinogenesis (85).

The O\textsuperscript{6} MeGua adduct can eventually lead to base mis-pairing during DNA replication thus introducing a change in base sequence in the daughter strand. For malignancy to take place, replication must occur prior to the alkylated base being removed from DNA. In the esophagus, repair is slow due to rapid metabolism of NMBA and consequent alkylation of DNA. The O\textsuperscript{6} MeGua adduct leads to the formation of two different mutations. The major mutation is the result of direct alkylation on single- or double-stranded DNA leading to a C:G→A:T transition. The second minor mutation is an A:T→G:C transition formed by a G:T mispair by the enzyme O\textsuperscript{6}-methyltransferase (81). The methyldiazonium ion can also bind to other macromolecules such as RNA and protein in a series of reactions (not shown).

The second pathway involves the hydroxylation of the methyl carbon in NMBA. This carbon eventually gets converted to benzyl alcohol, a benzylating agent, and formaldehyde. Benzyl alcohol can be converted to benzaldehyde thus leading to mutational events by the process described above. Benzaldehyde, benzoic acid, and benzyl alcohol are all metabolites that can be measured in studies of NMBA metabolism (16). Both metabolic pathways are active in rat esophageal cells, although hydroxylation of the methylene carbon (first pathway) far exceeds hydroxylation of the methyl carbon (second pathway) as the major pathway for NMBA metabolism in esophagus. This is supported in studies performed by Autrup and Stoner, who showed that levels of benzylation
in the rat esophagus were one-tenth that of methylation (84). In addition, benzaldehyde formation exceeded that of formaldehyde plus CO₂ by a factor of six, indicating that methylene hydroxylation was preferred. Levels of 7-methylguanine and O⁶-methylguanine are considerably higher in the esophagus than in any other tissue in animals treated with NMBA (29). Treatment of rats with NMBA can result in depletion of the O⁶-methylguanine transferase activity leading to an accumulation in the levels of O⁶-methylguanine. Rat microsomes exhibit a 70-fold higher capacity for metabolizing NMBA than human microsomes. In addition, DNA methylation in cultured explants of rat esophagus treated with NMBA is much higher than in explants of human esophagus (84). This leads one to question the importance of NMBA as a carcinogen for the human esophagus.

1.2.3. Pathology and histopathology of NMBA-induced rat esophageal tumorigenesis

The esophagus is a straight muscular tube that runs from the pharynx to the stomach (86, 87). It lies dorsal to the larynx and the anterior end of the trachea in the neck region and is left of the trachea in the thorax region. Although mostly lying in the thorax region, the end of the esophagus lines up towards the middle of the stomach. The length of the esophagus varies with the type of species. In man, it is approximately 25 cm long while in rats it ranges from 5-7 cm. Despite some degree of modification in lower animals, the histological appearance of the esophagus is typical as shown in Figure 1.3. Basically, the esophagus is divided into three layers, the muscle, mucosa, and
Figure 1.3: Histologic diagram of rat esophagus in longitudinal section. Modified from Craddock, 1993 (13).
stratified squamous epithelium. The muscle is composed of a circular, longitudinal and fibrous coat. The mucosa consists of lamina propria, muscularis mucosa, and submucosa layers. The squamous epithelial layer is composed of a basal layer, a granular layer, and a spinous layer. The basal epithelial cells of the esophagus lie over the basement membrane and have replicating potential. During the replication process, one or both of the daughter cells move upward into the granular layer. As they move upward, the cells differentiate forming large spinous cells and polygonal granular cells. These cells become flatter in shape eventually forming squamous cells which may or may not produce keratin. The glandular surface of the esophagus can protect itself against food consumption. In rats and horses, the epithelial surface is keratinized while in mice and other rodents the cells produce mucous instead of keratin. Keratinization is rare in man, monkeys, cats and dogs unless it is caused by trauma. Production of mucus, which is secreted into the lumen through small orifices, protects the esophagus. The lamina propria is the layer just below the stratified squamous epithelium. It contains loose connective tissue, blood vessels, lymphatics, and scattered inflammatory cells. Thin collagen and fine elastic fibers surrounded by numerous lymphocytic cells are also present. The muscularis mucosa is the layer that separates the laminal propria from the submucosa. This layer is composed of circular and longitudinal smooth muscle and also contains loose connective tissue, blood vessels, numerous lymphatics, autonomic nerve fibers, ganglion cells, adipose tissue, and scattered mononuclear inflammatory cells. The submucosa, the layer under the muscularis
mucosa, is composed of dense fibrous connective tissue and coarse elastic and collagen fibers. In humans, this layer is highly sensitive to certain foods and gastric juices produced by acid reflux from the stomach. The muscle layers below the submucosa are responsible for the contraction of the esophagus. Contraction of the longitudinal muscle shortens the esophagus while contraction of the circular muscle constricts the lumen. The upper end of the esophagus is closed off by the pharyngeal-esophageal sphincter thus preventing regurgitation into the pharynx while the esophageal-sphincter closes off the lower end of the esophagus.

The dose schedule used to initiate NMBA-induced esophageal tumorigenesis can cause extensive damage, and restorative hyperplasia to the esophagus. The histopathological and morphogenetic changes that occur during NMBA-induced esophageal tumorigenesis in the rat range from a normal orientation and progress to stages of hyperplasia, dysplasia, papillomas and eventually carcinoma in situ (Figure 1.4). This multistage process closely mimics the progression of esophageal SCC seen in humans. Under normal conditions, the basal and squamous layers comprise a uniform shape and undergo cell division at a normal rate. The mitotic index (MI), defined as the percentage of basal cells that are dividing in the basal epithelial layer is about 20% (83). Basal cells, which are approximately 1 to 2 cell layers in thickness, undergo replication and divide into two daughter cells. One cell replaces the preexisting basal cell while the other cell differentiates into a squamous cell. At the same time, squamous cells replace old keratin with new keratin. During the process of
Figure 1.4: Classification of microscopic histopathological and morphogenetic changes that occur during NMBA-induced esophageal tumorigenesis in the F344 rat esophagus. Normal esophageal epithelium (A); epithelial hyperplasia (B); low-grade dysplasia (C); high-grade dysplasia (D); squamous cell papilloma (E); and carcinoma *in situ* (F).
hyperplasia, a number of changes occur that deviate from normal. First, the mitotic index increases from 18-20% to approximately 35-40% (73). In addition, basal cells within the basal layer proliferate at a higher rate than seen under normal conditions. As a result of this, keratinization occurs at a much higher rate. In addition, the basal cell layer increases to more than 3 cell layers in thickness. Cell orientation is somewhat disorganized during stages of hyperplasia. Moderate to severe hyperplastic cells tend to progress towards dysplasia. During dysplasia, the basal layer becomes more thickened and there is a loss of normal cell orientation. The mitotic index is around 60-70%, so cell proliferation occurs at a higher rate. The cells exhibit an increased nuclear:cytoplasmic ratio, a loss of polarity, and frequent mitoses that tends to be abnormal in shape. Dysplasia can be classified as mild, moderate, or severe. Mild dysplasia occurs when abnormal cells are limited to the basal third of the mucosa, moderate when the cells extend to the middle third, and severe when the cells extend towards the upper third of the epithelium. Dysplastic cells continue to proliferate and eventually form into papillomas. Papillomas are benign neoplasms that tend not to metastasize and are not invasive. The lesion is characterized by surface papillary acanthotic squamous epithelium and a branched core of fibrovascular tissue. The epithelium shows no signs of atypia. Papillomas, however, tend to progress into carcinomas. Well-differentiated squamous cell carcinomas appear to have visible intercellular bridges, individual cell keratinization, and squamous pearl formation. In contrast, poorly differentiated squamous cell carcinomas tend not to contain any intercellular
bridges and squamous pearls. However, a small amount of keratinization is present which helps to establish a diagnosis. Carcinomas are characterized by their ability to invade and metastasize.

1.2.4. Molecular events associated with NMBA-induced rat esophageal tumorigenesis

A few genetic alterations have been observed as the result of NMBA-induced esophageal tumorigenesis in rats (Figure 1.5). Methylation products from O6-methylguanine adduct formation cause G:C→A:T transition mutations in position 2 of codon 12 of the Ha-ras gene. Recent studies have shown a high frequency of Ha-ras codon 12 mutations in papillomas of the rat esophageal animal model (88). This mutation occurs with low frequency in preneoplastic tissues, but is seen in approximately 80% of esophageal papillomas. The mutation of position 2 of codon 12 is identified as a “hot spot”. Other molecular events in esophageal papilloma development include a 2.8-fold elevation in cyclin E1 mRNA, elevated levels of cyclin E mRNA, and extensive G1 cyclin immunostaining indicating that cell cycle regulation is altered during rat esophageal tumorigenesis (16). Increased levels of proliferating cell nuclear antigen (PCNA), deregulated expression of transforming growth factor B1 and altered localization of E-cadherin and α-catenin are also seen in esophageal tumors in the rat. Studies by Carlton et. al have demonstrated that elevated levels of COX-2 in esophageal papillomas and preneoplastic esophageal tissues when NMBA is administered when NMBA is administered once a week for 15
Figure 1.5: Molecular alterations in the rat esophagus. Modified by Stoner and Gupta (16).
weeks (89). However, COX-2 mRNA levels increased in papilloma tissues only when NMBA was given 3 times a week for 5 weeks. Furthermore, Chen et. al found similar results in iNOS mRNA levels (90). Elevated levels of prostaglandin E$_2$ have also been found in NMBA-induced papillomas.

Human esophageal papillomas and carcinomas also undergo a number of molecular changes (9, 29, 91). The tumor suppressor gene, p53 undergoes a variety of molecular changes leading to altered DNA replication and repair, cell proliferation, and apoptosis. Mutations in p53 can also disrupt the G1/S cell cycle thus leading to loss of cell cycle control. Once p53 is inactivated or disrupted, alterations in oncogene function can result leading to a deregulation of cell signaling cascades. Methylation and/or loss of p15, p16MST1, and RAR$\beta$ gene expression and reduced Rb expression levels are also seen indicating losses in tumor suppressor gene function. Studies with cell lines have shown that altered ras gene expression is associated with human esophageal SCC. Many growth factors are overexpressed in human esophageal SCC. These include epidermal growth factor (EGF), transforming growth factor alpha (TGF$\alpha$), and epidermal growth factor receptor (EGFR) (16). Cell proliferation proto-oncogenes, cyclin D1 as well as HST-1 and INT-2, tend to be amplified. c-myc, iNOS, COX-2, hTERT, BMP-6 and cytoplasmic $\beta$-catenin are amplified in a small percentage of esophageal squamous cell carcinomas. Loss of heterozygosity on chromosomes 1p, 3p, 4, 5q, 9, 11q, 17q, and 18q are frequently seen in esophageal SCC. Different chromosome regions on genes have been identified as being associated in the development of esophageal SCC. Specific
chromosome regions and genes include chromosome 8q22 (FEZ-1) chromosome 3p21 (DLC1) and chromosome 17q25 [tylosis esophageal cancer (TOC) gene]. The first two genes are tumor suppressor genes while the last gene is associated with the development of esophageal cancer by the age of 70 in >90% of affected individuals. Few studies have reported molecular changes that occur early on in esophageal SCC development. However, there is loss of p53 gene function in the early stage (16).

Genetic polymorphisms in carcinogen metabolizing enzymes have been reported to increase an individual’s susceptibility to develop esophageal SCC (16, 92, 93). Studies of residents in Linxian, China found higher levels of CYP2E1 enzyme activity among subjects with esophageal dysplasia. Another study found reduced activity of the glutathione S-transferase M1 gene (92, 93). Data relating genetic polymorphisms to esophageal SCC still warrants further investigation.

1.3. Chemoprevention of esophageal cancer

Cancer chemoprevention is defined as the prevention of cancer by use of dietary or synthetic chemicals (94, 95, 96). Sporn et al. defined chemoprevention as the use of specific natural or synthetic chemical agents to reverse, suppress or prevent the carcinogenic process to invasive cancer (96). While there are generally two types of subject categories for chemoprevention, the projected target populations for cancer chemoprevention are comprised of high-risk groups, such as those individuals who: (a) engage in risk-taking behavior or lifestyles (e.g. smokers and users of snuff); (b) received occupational exposure to known
carcinogens (e.g. asbestos workers); (c) are known to be genetically predisposed to the development of cancer (e.g. individuals with familial colonic polyposis, etc.); (d) possess premalignant lesions (e.g. oral leukoplakia in snuff users); (e) survivors of primary cancers with a high degrees of recurrence or a high tendency towards formation of second primary tumors; and (f) cancer survivors that received chemotherapy and/or radiation therapy. Ideal qualities that go into a chemopreventive agent include low cost, high efficacy, human acceptance, known mechanism, little or no toxicity, and oral consumability. Chemopreventive agents can be classified as inhibitors of carcinogen formation, blocking agents, and suppressing agents (95, 97). Inhibitors of carcinogen formation represent of a minority of chemopreventive compounds. Basically, they are involved in preventing the formation of nitrosamines from secondary amines and nitrites when placed in an acidic environment. Blocking agents are inhibitors of tumor initiation while suppressing agents are involved at inhibiting tumor promotion / progression. Blocking agents work at preventing the formation of tumors during the initiation phase of tumor development. Their mechanism of action falls under one of three major categories; inhibiting cytochrome P450 enzymes, inducing cytochrome P450 enzymes, and induction of phase II enzymes (Table 1.4). The last category is broken down further into specific pathways in which phase II enzymes are involved in carcinogenesis. One of the most well studied and potent inhibitors of cytochrome P450 enzymes are the isothiocyanates. Studies by Stoner and al. showed that administration of dietary phenethyl isothiocyanate
(PEITC) at a dose of 3 mmol/kg diet was effective at inhibiting NNK-induced lung tumors by 50% and completely inhibiting NMBA-induced esophageal tumorigenesis (68, 97). In addition, 6-phenylhexyl isothiocyanate (PHITC) reduced NNK-induced lung tumorigenicity by >80% in the strain A mouse model (67). Furthermore, the isothiocyanates, PEITC and PPITC, were both effective at reducing O\(^6\)-methylguanine adduct formation in DNA. Some isothiocyanates such as PHITC actually enhanced esophageal tumorigenesis (98). Another mechanism in which blocking agents work is by inducing cytochrome P450 enzymes. Agents under this mode of action can either act by increasing metabolic activation of carcinogens in non-target tissues or enhance the oxidative detoxification of carcinogens at any tissue site. It is important to note that agents which act by increasing metabolic activation of certain P450 enzymes may promote tumorigenesis at other organ sites thus giving a rationale why specific compounds such as indole-3-carbinol may possess enhancing activity (97). Blocking agents also act by inducing phase II detoxification enzymes. These agents are preferred over cytochrome P450 inducers because of they lack the ability to produce cancer. Examples of these agents include sulforaphane and butylated hydroxyanisole (BHA).

In addition to blocking agents, there are agents that inhibit the sequence of events associated with tumor promotion/progression; i.e., suppressing agents. Classification of suppressing agents is somewhat difficult since the sequence of events associated with tumor promotion/progression is not entirely understood. Examples of suppressing agents as well as their mechanisms of action are listed
in Table 1.4. Suppressing agents used in our laboratory include NSAID’s and other COX inhibitors (89), DFMO, polyphenols (69), retinoids (99), sulindac sulfone (100), and iNOS inhibitors (90).

1.4. Food components and esophageal cancer prevention.

Epidemiological data suggest an inverse relationship between consumption of fruit and vegetables and the occurrence of several types of cancer (101-103). Interestingly enough, 40% of all human cancers are associated with dietary factors. In particular, the incidence of SCC of the esophagus has been linked to diets deficient in fruit and vegetables (38). A large number of studies have reported that vitamins and minerals such as folate, beta-carotene, specific carotenoids, vitamin A, C, and E, fiber, and other components contained in fruits and vegetables have been implicated with the prevention of certain types of cancers (94). Studies conducted by Block et al. reported that persons with a low fruit and vegetable intake have twice the risk of developing cancer at most sites compared to those with a high intake after controlling for confounding factors (104).

FDA studies have concluded that the incidence of esophageal SCC is inversely related to intake of vitamin-C rich foods. In general however, the associations seem to be weaker for individual nutrients than for whole fruits and vegetables.

A number of chemopreventive agents found in foods have been shown to be effective against N MBA-induced esophageal tumorigenesis in the rat model. For example, the plant polyphenol, ellagic acid, reduced N MBA-induced
esophageal tumorigenesis (64, 65) in F344 rats. When administered in the diet at a concentration of 0.4 and 4g/kg, ellagic acid caused a 21-55% reduction in the number of esophageal tumors after 20 and 27 weeks of the study. Ellagic acid was also effective at inhibiting preneoplastic and neoplastic lesions when examined by histopathology. In addition, ellagic acid was effective at inhibiting metabolites from NMBA in cultured rat esophagus (105), and in reducing the metabolism of NMBA and DNA-induced adduct formation in esophageal but not liver microsomes (106). Phenethyl isothiocyanate (PEITC), which is found in cruciferous vegetables, caused a 99% reduction in NMBA-induced esophageal tumorigenesis when administered at a concentration of 3.0 µmol/g or greater in the diet (68). It was also shown to be effective at reducing DNA-adduct formation \textit{in vivo}. This compound, however, had no effect in the anti-promotion/progression scheme (76, 118). Diallyl sulfide, a thioester contained in garlic, was also shown to completely inhibit NMBA-induced preneoplastic lesions and (119) esophageal cancer, however, it was only effective at reducing NMBA metabolism in liver and not in esophageal microsomes (63). Green tea, abundant in polyphenols, was shown to have both blocking and suppressing activity when administered with NMBA (107). A complete list of other known chemopreventives found in food are listed in Table 1.4.
<table>
<thead>
<tr>
<th>Mechanism of action</th>
<th>Example of agent(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inhibitors of carcinogen formation</strong></td>
<td></td>
</tr>
<tr>
<td>Reductive acids</td>
<td>Absorbic acid</td>
</tr>
<tr>
<td>Phenols</td>
<td>Caffeic acid, ferulic acid, gallic acid</td>
</tr>
<tr>
<td>Sulfhydryl compounds</td>
<td>N-acetylcysteine</td>
</tr>
<tr>
<td>Amino acid</td>
<td>Proline, thioproline</td>
</tr>
<tr>
<td><strong>Categories of anti-initiating (blocking) agents</strong></td>
<td></td>
</tr>
<tr>
<td>Inhibition of cytochrome P450</td>
<td>Dithiocarbamates, ellagic acid, diallyl sulfide, isothiocyanates</td>
</tr>
<tr>
<td>Induction of cytochrome P450</td>
<td>Indole-3-carbinol, β-naphthoflavone</td>
</tr>
<tr>
<td>Induction of phase II enzymes</td>
<td></td>
</tr>
<tr>
<td>Glutathione S-transferase</td>
<td>Allyl sulfides, dithiolethiones, isothiocyanates</td>
</tr>
<tr>
<td>UDP-glucuronyltransferase</td>
<td>Polyphenols</td>
</tr>
<tr>
<td>Glutathione transferase</td>
<td>Selenium</td>
</tr>
<tr>
<td>Scavenge electrophiles</td>
<td>Ellagic acid, N-acetylcysteine</td>
</tr>
<tr>
<td>Scavenge free radicals</td>
<td>Sodium thiosulfate, polyphenols, vitamin E</td>
</tr>
<tr>
<td>Increase overall levels of DNA repair</td>
<td>Vanillin</td>
</tr>
<tr>
<td>Increase poly (ADP-ribosyl) transferase</td>
<td>N-acetylcysteine</td>
</tr>
<tr>
<td>Suppress error-prone DNA repair</td>
<td>Protease inhibitors</td>
</tr>
<tr>
<td><strong>Categories of anti-promotion/progression (suppressing) agents</strong></td>
<td></td>
</tr>
<tr>
<td>Inhibition of polyamine metabolism</td>
<td>DFMO, polyphenols, substituted putrescines</td>
</tr>
<tr>
<td>Induce terminal cell differentiation</td>
<td>Calcium, retinoids, vitamin D₃</td>
</tr>
<tr>
<td>Modulate signal transduction</td>
<td>Glycyrrhetinic acid, NSAID’s, polyphenols, retinoids</td>
</tr>
<tr>
<td>Modulate hormonal / growth factor activity</td>
<td>NSAID’s, retinoids, tamoxifen</td>
</tr>
<tr>
<td>Inhibit oncogene activity</td>
<td>Genistein, NSAID’s, monoterpenes</td>
</tr>
<tr>
<td>Promote intracellular communication</td>
<td>Cartenoids, polyphenols, retinoids</td>
</tr>
<tr>
<td>Restore immune response</td>
<td>NSAID’s, selenium, vitamin E</td>
</tr>
<tr>
<td>Induce apoptosis</td>
<td>Butyric acid, genistein, selenium, sulindac sulfone, retinoids</td>
</tr>
<tr>
<td>Correct DNA methylation imbalances</td>
<td>Folic acid, choline, methionine</td>
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<tr>
<td>Inhibit basement membrane degradation</td>
<td>Protease inhibitors</td>
</tr>
<tr>
<td>Inhibit arachidonic acid metabolism</td>
<td>Glycyrrhetinic acid, N-acetylcysteine, NSAID’s, polyphenols</td>
</tr>
</tbody>
</table>

Table 1.4: Classification and examples of cancer chemopreventive agents based on mechanism of action. Modified from Morse and Stoner, 1993 (95).
1.5. Inhibitors of polyamine synthesis

Polyamines are important for normal cell growth and function. Most of them are abundant in cells as outlined in Table 1.5. The ability of polyamines to bind to nucleic acids, in particular DNA, and their ability to stimulate cell growth makes them important for the process of carcinogenesis (95). Polyamine biosynthesis is divided into a series of steps as outlined in Figure 1.6. The first step is the formation of the amine, putrescine. This reaction occurs by the decarboxylation of ornithine and is catalyzed by the enzyme ornithine decarboxylase (ODC). Although ornithine is readily available in the plasma, it can be synthesized from arginine. The concentration of arginine, which is present in extrahepatic tissues, ensures the availability of ornithine. After putrescine is formed from ornithine it is converted to spermidine. In order to carry out this reaction, an aminopropyl moiety derived from methionine after its conversion to 5-adenosylmethionine is added to spermidine. This reaction is catalyzed by the aminopropyltransferase enzyme, spermidine synthase. Another aminopropyl group from decarboxylated S-adenosylmethionine is needed to convert spermidine into spermine. This reaction is catalyzed by the enzyme, spermine synthase. In general, the reactions using spermidine and spermine synthase are irreversible while putrescine formation can occur in either direction. In addition, spermidine and spermine also produce N\(^1\)-acetylspermidine and N\(^1\)-acetylspermine, respectively. The function of these by products is not entirely known.
Table 1.5: Early known polyamines and their occurrence in cells. Modified from Heby, 1981 (108).

<table>
<thead>
<tr>
<th>Polyamine</th>
<th>Structure</th>
<th>Occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putrescine</td>
<td>NH₂(CH₂)₃NH₂</td>
<td>Widespread</td>
</tr>
<tr>
<td>Cadaverine</td>
<td>NH₂(CH₂)₅NH₂</td>
<td>Restricted</td>
</tr>
<tr>
<td>Spermidine</td>
<td>NH₂(CH₂)₃NH(CH₂)₄NH₂</td>
<td>Widespread</td>
</tr>
<tr>
<td>Spermine</td>
<td>NH₂(CH₂)₃NH(CH₂)₄NH(CH₂)₃NH₂</td>
<td>Widespread</td>
</tr>
</tbody>
</table>
Figure 1.6: Polyamine biosynthesis in mammalian tissues.
Figure 1.7: Chemical structure of DFMO.
There is a great deal of evidence that elevated polyamine levels are associated with cell proliferation (108). Early studies have shown that exogenous polyamines are necessary for maximal growth rate suggesting that polyamines play a role in the process of cellular growth (110). Research by Heby, Marton, et al. demonstrated that levels of cellular putrescine, spermidine, and spermine significantly increase as cells progress from the G1 to M phase of the cell cycle (108). This increase is mainly due to elevated levels of the enzyme ODC. As mentioned previously, ODC is the rate-limiting enzyme responsible for the catalyzation of L-ornithine (and to some extent L-lysine) to yield putrescine and carbon dioxide. Mammalian ODC is a pyridoxal phosphate dependent enzyme and was discovered in 1968 (109). It is a growth related protein, thus its activity is greatly enhanced by stimuli such as certain hormones, drugs, tissue regeneration, and growth factors. ODC has an extremely short molecular half-life of only 10-20 minutes and is present in small amounts within quiescent cells. Overexpression of ornithine decarboxylase (ODC) plays an important role in both normal cellular proliferation and the growth and development of tumors. High basal levels of polyamines in many epithelial tumors are exhibited when ODC is aberrantly regulated in tumor cells. This, in turn, can result in the up-regulation of ODC expression by oncogenes such as c-myc, v-src, v-raf, or activated ras or rhoA proteins (108). Subsequently, many studies have reported a correlation between ODC expression and polyamine levels in tumors and the tumor progression stages of various cancers. For example, high levels of urinary metabolites of polyamines are found in many cancer patients, and an increased
ratio of spermidine to spermine in mouse hepatomas (109). Levels of ODC are increased in adenomatous polyps of the colon that were in the process of shifting to malignancy. Poorly differentiated SCC have several-fold higher levels of ODC than well differentiated SCC in patients with advanced head and neck cancers. In astrocytoma patients, the contents of ODC and putrescine increased with malignancy. Increased levels of polyamines were found in adenocarcinomas of the human thyroid, prostate and breast tissue, and in urine among patients with lymphomas (both Hodgkin's and non-Hodgkin's disease). The most convincing evidence for the role of ODC in tumorigenesis comes from studies in mouse skin using chemical carcinogenesis in which diverse tumor promoters were shown to induce ODC activity (110).

With all the information surrounding ODC's effect on polyamine levels, finding inhibitors that would block or inhibit the enzyme could serve as a viable target for chemical intervention. Studies have shown that using amine derived inhibitors similar in structure to ornithine would be the most effective at blocking the action of ODC (111). ODC-catalyzed inhibitors work to inhibit ODC through a variety of mechanisms. They act by blocking the cofactor of the enzyme, or the substrate, ornithine, or the product, putrescine. Although each inhibitor alone, or in combination, was effective to a certain degree, they were not specific and most were reversible. The most powerful blockers of ODC activity are the mechanism-based irreversible inhibitors of which difluoromethylornithine (DFMO) is the most studied (111). The structure of DFMO is shown in Figure 1.7. DFMO mimics the natural substrate of ODC, ornithine, but remains after activation, covalently
bound to ODC. DFMO decreases the concentration of putrescine and spermidine while leaving the concentration of spermine unchanged in animal cells. When the levels of polyamines are reduced, the rate of cell proliferation decreases. The mechanism by which DFMO prevents cancer involves more than inhibition of cell proliferation since in vivo studies have shown that DFMO affects the transition of noninvasive tumors to invasive cancers, thus acting late in the scheme of chemical carcinogenesis (111). Some studies indicate that DFMO may also have pro-apoptotic properties (108). Subsequently, the expression of genes affecting tumor invasion, including matrix metalloproteinases, are dependent on polyamines inhibited by DFMO in several cell types (111). DFMO interferes with polyamine biosynthesis, and this depletion of cellular polyamines depletes DNA synthesis by reducing the rate of DNA elongation.

Several studies have been conducted in which DFMO has been shown to be an effective agent against a number of cancers both in vivo and in human clinical trials. In vivo studies have shown that DFMO inhibits esophageal cell proliferation by activating apoptosis when given before and after carcinogen treatment in zinc-deficient rats (112). DFMO lowered TPA-induced ODC activity in mouse skin when given topically, parenterally or administered in the drinking water (113). It also suppresses intestinal polyamine content in the Min mouse model of colon cancer (114) and is highly effective as a preventive agent against azoxymethane induced colon cancer in rats (111). Furthermore, DFMO reduces chemically induced rat urinary bladder carcinogenesis (111). And finally, DFMO
inhibits DMBA-induced rodent mammary carcinoma (115). Chemoprevention trials using DFMO as a possible chemopreventive have been conducted in breast, Barrett’s esophagus, cervix, prostate, nonmelanoma skin cancer, and colon (111).

DFMO is well tolerated in humans with minimal toxicity, but has been associated with ototoxicity on prolonged administration (111). It is an excellent candidate for use as a chemopreventive agent especially in high-risk individuals to prevent the development of neoplasia. In view of experimental data showing the inhibitory effect of DFMO in a number of cancers, we wanted to determine if DFMO was effective in preventing NMBA-induced esophageal tumorigenesis in rats. In a preliminary study, our laboratory had shown DFMO to be an effective inhibitor of \(N\)-nitrosomethylbenzylamine (NMBA)-induced tumorigenesis in the rat esophagus when administered before, during and after carcinogen treatment. Specifically, DFMO was shown to inhibit NMBA-induced esophageal tumors by 40%. In order to evaluate the potential post-initiation effects of DFMO, an animal study was conducted in which we administered DFMO following the completion of carcinogen treatment. The goal of this study was to determine if DFMO was an effective agent for rats that were pre-initiated with NMBA.

1.6. Proposed research

Accordingly, in addition to chemoprevention approaches, our laboratory has recently taken a “food based” approach to the prevention of esophageal cancer in rodents. We found that the administration of freeze-dried strawberries and black raspberries, at concentrations of 5 and 10% in a synthetic diet,
produced significant decreases in NMBA-induced esophageal tumors in rats (72-74). Thus both berry types were effective when given in the diet before, during and after NMBA treatment, and also when administered only after NMBA exposure. The berries were also effective at reducing the number of preneoplastic lesions in the rat esophagus of NMBA treated animals (72-73). In addition, studies by Kresty et. al found that black raspberries have been shown to reduce cell proliferation rates by positively staining the cells with PCNA (73). Furthermore, black raspberries have been shown reduce formation of O\textsuperscript{6}\text{-methylguanine adducts from NMBA in rat esophagus (73).} Studies in the colon by Harris et. al demonstrated the ability of black raspberries to reduce oxidative stress by measuring levels of 8-hydroxy-deoxyguanosine in the urine of rats treated with azoxymethane (116). In view of the preventative effects observed with strawberries and black raspberries in the rat esophagus, we decided to evaluate freeze-dried blueberries for their potential to inhibit esophageal tumorigenesis. Blueberries, like strawberries and black raspberries, contain multiple known chemopreventive agents (Table 1.6). In addition, blueberries possess higher antioxidant activity and anthocyanin levels than strawberries and some other berry types (117). The second chapter in this thesis describes the results of our study to evaluate freeze-dried blueberries as an inhibitor of esophageal cancer in rodents. The latter part of the chapter details an adduct study demonstrating whether freeze-dried blueberries were effective at inhibiting DNA adducts produced from NMBA exposure.
Instead of using a food-based approach at inhibiting tumors, the next chapter discusses the use of a synthetic agent at inhibiting esophageal tumorigenesis. Specifically, this chapter outlines a study in which a known chemopreventive, difluoromethylornithine (DFMO), a competitive inhibitor of ornithine decarboxylase is analyzed for its effects on esophageal tumorigenesis in rats treated with NMBA is analyzed in a post-initiation scheme.

Since of berries, in particular, black raspberries, inhibit the initiation and promotion / progression stages of tumor development (73,121), we wanted to examine whether they posses some therapeutic value. The fourth chapter describes the details of this study in which black raspberries were tested for their potential therapeutic effects in rats that had already fully developed tumors in their esophagus at the time of berry administration.
<table>
<thead>
<tr>
<th>Components</th>
<th>Black Raspberries</th>
<th>Strawberries</th>
<th>Blueberries</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Minerals</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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</tr>
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<tr>
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<tr>
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<td></td>
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</tr>
<tr>
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<td>&lt;1.00</td>
<td>&lt;1.00</td>
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<tr>
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<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>β-carotene</td>
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<td>0.29</td>
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<td>Folic acid</td>
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<td>0.05</td>
</tr>
<tr>
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<td>267.00 I.U.</td>
<td>483.00 I.U.</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>415.00</td>
<td>298.00</td>
<td>19.30</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>15.12</td>
<td>4.95</td>
<td>7.68</td>
</tr>
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<td><strong>Sterols</strong></td>
<td></td>
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<td>β-sitosterol</td>
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<td>85.70</td>
</tr>
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<td>&lt;3.00</td>
</tr>
<tr>
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<td>&lt;3.00</td>
</tr>
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</table>

Table 1.6: Components of different berry types used in chemoprevention studies. All samples are reported in mg/100g sample, except selenium levels reported in mcg/100g.
CHAPTER 2

THE EFFECT OF FREEZE-DRIED BLUEBERRIES ON N-NITROSOMETHYLBENZYLAMINE-INDUCED TUMORIGENESIS IN THE F-344 RAT ESOPHAGUS

2.1 Abstract

Previous studies in our laboratory have demonstrated the inhibitory effects of freeze-dried strawberries and black raspberries on N-nitrosomethylbenzylamine (NMBA)-induced tumorigenesis in the rat esophagus. In addition, organic extracts from strawberries and black raspberries were shown to inhibit benzo[a]pyrene-induced transformation of Syrian hamster embryo (SHE) cells in vitro and were shown to have an effect at reducing AP-1, NFκB and VEGF expression in CD41 mouse epidermal cells. In the present study, we evaluated blueberries for their ability to inhibit NMBA tumorigenesis in the rat esophagus. Blueberries, like strawberries and black raspberries, contain multiple cancer preventive agents, and are among the most heavily consumed berries in the American diet. They differ from strawberries and black raspberries however, in that they contain only small amounts of the chemopreventive agent, ellagic acid. Two weeks prior to NMBA treatment, animals were placed on a control diet or diets containing 5 and 10% freeze-dried blueberries. NMBA treatment was
once per week for 15 weeks. At 25 weeks, 5 and 10% blueberries produced no
significant differences in tumor incidence or size when compared to NMBA-
treated controls. In addition, a short-term bioassay found that blueberries did not
reduce the formation of NMBA-induced O\(^6\)-methylguanine adducts in esophageal
DNA when fed at 10% of the diet. Blueberries, therefore, appear to lack
components that inhibit the initiation and progression of NMBA-induced
tumorigenesis in the rat and the metabolism of NMBA into DNA damaging
species.

2.2. Introduction

Esophageal cancer ranks eighth in cancer incidence worldwide and is the
fifth most common cause of cancer death (1). Current data estimates
approximately 13,000 deaths from esophageal cancer in 2003, while it is
estimated that 13,900 new cases will occur in 2004 (2, 3). In the United States,
esophageal cancer is relatively uncommon, but it is associated with a 5-year
survival rate of only 12% from the disease at all stages while 75% die within a
year of diagnosis (4, 5, 6). Moreover, mortality estimates represent 95% of the
expected incidence in 2003 (1). Regions of the world that have the highest
incidence rates include China and other Asian countries, the Transkei region of
South Africa, Iran, France, the Caribbean and Puerto Rico (6, 7). Esophageal
cancer is of two major types; squamous cell carcinoma (SCC) which occurs
principally in the middle and distal portion of the esophagus and adenocarcinoma
which arises at the distal portion of the esophagus. Approximately 90% of all
esophageal cancers worldwide are squamous cell carcinomas.
Adenocarcinomas, which arise from a preneoplastic lesion termed Barrett's esophagus, account for 5% of esophageal malignancies and their rate of occurrence is increasing rapidly in the United States (8). The other 5% of esophageal cancers are mesenchymal tumors (9).

Among the risk factors associated with the development of SCC of the esophagus include tobacco and alcohol consumption, salt-cured, salt-pickled and moldy foods, dietary deficiencies in certain vitamins and minerals, and consumption of hot and spicy foods (10, 11, 12). Some of these products are frequently contaminated with N-nitrosamine carcinogens and/or fungal toxins. Extensive research in China and South Africa has suggested that N-nitroso compounds, including N-nitrosomethylbenzylamine (NMBA), and their precursors are probable etiological factors for esophageal SCC in these high incidence areas (13, 14). In view of these exposures, one approach to the prevention of esophageal SCC is through changes in lifestyle, including avoidance of tobacco and alcohol and an increase in vitamins and mineral consumption in the diet. Additional benefits may be realized with the elimination of high salt foods that may be contaminated with microbial toxins, nitrosamines and their precursors. Chemoprevention, to address factors associated with the etiology and progression of the disease, is another viable approach. Indeed, using a rat model of esophageal SCC in which tumors are induced with the nitrosamine, NMBA, our laboratory and others have identified multiple chemopreventive agents for the esophagus (15). Moreover, clinical trials have identified some
chemopreventive agents that appear to be useful for reducing the risk for development of esophageal SCC in humans (15).

Epidemiological data suggest an inverse relationship between consumption of fruit and vegetables and the occurrence of several types of cancer (16). In particular, the incidence of SCC of the esophagus has been linked to diets deficient in fruit and vegetables (14). Accordingly, in addition to chemoprevention approaches, our laboratory has recently taken a “food based” approach to the prevention of esophageal cancer in rodents. We found that the administration of freeze-dried strawberries and black raspberries, at specific concentrations in the synthetic diet, produced significant decreases in NMBAd-induced esophageal tumors in rats (17, 18, 19). Specifically, freeze-dried black raspberries caused a 39% and 49% reduction in esophageal tumor multiplicity when administered in the diet before, during and after treatment of rats with carcinogen. When administered only after carcinogen treatment, freeze-dried black raspberries caused a 37% and 31% reduction in tumors, respectively. Similarly, freeze-dried strawberries caused a 24% and 56% reduction in esophageal tumor multiplicity when administered in the diet before, during and after treatment of rats with carcinogen. Thus, both berry types were effective in an anti-initiation and post-initiation scheme. The berries were also effective at reducing the number of preneoplastic lesions in the rat esophagus of NMBAd treated animals (18, 19). Furthermore, Kresty et. al found that black raspberries reduced cell proliferation rates by positively staining the cells with PCNA (19). Studies in the colon by Harris et al. demonstrated the ability of black raspberries
to reduce oxidative stress by measuring levels of 8-OH deoxoguanosine in the urine of rats treated with azoxymethane (20). Short-term studies with Stoner et al. showed that black raspberries inhibit the formation of the promutagenic adduct O⁶-methylguanine (O⁶-meGua) by 73 and 80% respectively, after a single dose of NMBA at 0.25 mg/kg (19). Further studies have demonstrated that feeding 5% black raspberries also significantly inhibited adduct formation by 64% when 0.50 mg/kg of NMBA was given (19). Extracts isolated from strawberries and black raspberries were also shown to inhibit benzo(a)pyrene-induced transformation of Syrian hamster embryo (SHE) cells in vitro (21). In addition, extracts from black raspberries were shown to be effective at reducing AP-1 and NFκB expression in CD41 mouse epidermal cells (22). In view of the preventative effects observed with strawberries and black raspberries in the rat esophagus, we decided to evaluate freeze-dried blueberries for their potential to inhibit NMBA-induced esophageal tumorigenesis. Blueberries, like strawberries and black raspberries, contain multiple known chemopreventive agents including vitamins A, C, E, folic acid, β-carotene, α-carotene, ellagic acid, ferulic acid, ascorbic acid, zeaxanthin, folate and lutein as illustrated in Chapter 1, Table 1.5 (19, 23). Blueberries also contain specific minerals such as calcium, copper, iron, potassium, selenium and zinc and sterols like beta sitosterol, campesterol, and cholesterol. However, it is important to note that blueberries contain lower quantities of vitamin E and folic acid, calcium, zinc, selenium, ellagic acid, ferulic acid, and β-sitosterol than black raspberries (20, 24). Blueberries were also shown to possess higher antioxidant activity than strawberries, black raspberries
and some other 22 other fruits and vegetables using the oxygen radical absorbance capacity assay (ORAC) (25, 26, 27). Dietary antioxidant compounds may aid in scavenging oxygen free radical damage in the body, and their consumption is reported to promote good health and decrease the risk of degenerative diseases such as cardiovascular diseases and various cancers. High antioxidant activity is also a common feature of many food-based chemopreventives (28). Perhaps the most health promoting compounds contained in blueberries are the anthocyanins. Anthocyanins are part of a widespread class of compounds called flavonoids (29). Anthocyanidins, the aglycone form of anthocyanins is highly concentrated in blueberries and is responsible for producing the intense bluish red color found in the fruit. Studies show significant positive relationships were reported between ORAC antioxidant capacity and the content of anthocyanins in blueberries (26,28,29). Anthocyanins may also have antineoplastic, anti-inflammatory and chemopreventive effects (28). Anthocyanins have been shown to inhibit mutagenesis by environmental carcinogenic heterocyclic amines in the Ames assay. Furthermore, they have been reported that they can act as antitumor agents both in vivo and in vitro (30-32). Therefore, anthocyanins not only posses antioxidant activity but also mediate other physiological functions related to cancer suppression.

In view of the experimental data suggesting the positive aspects from freeze-dried black raspberries and strawberries at inhibiting esophageal tumorigenesis, we decided to conduct an anti-initiation study to determine if
freeze-dried blueberries would inhibit esophageal tumorigenesis. In addition, we conducted a short-term study in which freeze-dried blueberries were examined to see if they had any effect at inhibiting the formation of the promutagenic adduct O\textsuperscript{6}-methylguanine (O\textsuperscript{6}-meGua) adduct.

2.3. Materials and Methods

2.3.1 Animals

Male F-344 rats, 5-6 weeks-of-age, were purchased from Harlan-Sprague Dawley (Indianapolis, IN). The animals were housed 3 per cage under standard conditions (20 ± 2°C; 50 ± 10% relative humidity; 12-hour light/dark cycles). Rats were maintained on modified AIN-76A diet containing 20% casein, 0.3% D,L-methionine, 52% cornstarch, 13% dextrose, 5% cellulose, 5% corn oil, 1% AIN vitamin mixture, 3.5% AIN salt mixture and 0.2% choline bitartrate (Dyets, Bethlehem, PA). Food and water were provided ad libitum throughout the bioassay. Hygienic conditions were maintained by twice weekly cage changes. Body weight and food consumption measurements were recorded weekly for the duration of the study.

2.3.2. Chemicals

N-nitrosomethylbenzylamine (NMBA), obtained from Ash Stevens, Inc. (Detroit, MI), was determined to be greater than 98% pure by reverse phase HPLC analysis. Dimethyl sulfoxide (DMSO) was purchased from Sigma Chemical Company (St. Louis, MO).

2.3.3. Diet preparation
Blueberries (*Vaccinium corymbosum*) of the Rubel variety were provided by Cherry Central, Inc. (Transverse City, MI). They were picked in September, 1999, washed and frozen at -20°C within 24 hours of the time of picking. In March, 2000, the frozen blueberries were shipped to Van Drunen Farms (Momence, IL) where they were held frozen -20°C until freeze-dried one week later. Freeze-drying was performed as described previously (17, 18). Briefly, the blueberries were washed and excess water drained. Using a Brown pulper-finisher equipped with a 0.02” screen, the berries were crushed into a puree. The puree was then poured to a depth of approximately 1-inch into metal trays and, using a Virtis freeze-drying unit, the berries were freeze-dried (lyophilized). The freeze-dried berries were then shipped frozen to The Ohio State University (Columbus, OH) where they were stored at -20°C before use in the experiment.

In addition, 100 gram samples of freeze-dried blueberries were analyzed for compounds with known cancer inhibitory effects by Covance Laboratories (Madison, WI).

According to previous studies reported by Stoner et. al, 5 and 10% berries were shown to possess an inhibitory effect against esophageal tumors (17). Therefore, these were the doses chosen for this study. On a weekly basis, the freeze-dried blueberries were mixed for 20 minutes in modified AIN-76A diet at concentrations of 5 and 10% using a Hobart mixer. The corn starch in the diet was reduced by 5 and 10% to provide a similar caloric intake in the berry diets and the control diet. On a weekly basis, 5 and 10% of blueberry diets and control diet was placed into glass feeding jars and fed to the animals.
2.3.4. Experimental Design: Anti-initiation bioassay

The effects of lyophilized blueberries on NMBA tumorigenesis in the rat esophagus were evaluated in a carcinogenesis bioassay using 95 animals (Table 2.1). One week after acclimation to the facility, five groups of animals were randomized and placed on AIN-76A diet or AIN-76A diets containing 5% and 10% blueberries. Rats were maintained on these diets for the duration of the 25-week bioassay. Two weeks following the initiation of the diets, three groups of rats (Groups 3-5) were administered s.c. injections of NMBA at a concentration of 0.25 mg/kg body weight, once per week for 15 weeks (Figure 2.1). Group 1 was treated with 20% dimethyl sulfoxide (DMSO) in water, the solvent for NMBA. Group 2 was fed the high dose of blueberries, 10%. Twenty-five weeks following initiation of NMBA treatment, rats were killed by CO₂ asphyxiation. The esophagus of each animal was opened longitudinally and the surface tumors were mapped, counted and measured. Lesions greater than 0.5 mm in diameter were considered to be tumors. Assuming a spheroid shape, a volume estimate for each papilloma was calculated (33). The esophagi were then fixed in 10% neutral buffered formalin (NBF) and processed for histopathology. Multiple H&E stained slides were prepared from the esophagi of five rats per group and used for the quantification of preneoplastic lesions (i.e., hyperplasia, simple leukoplasia and dysplastic leukoplasia). In addition, sections of the liver, colon, stomach, kidney, spleen, heart, and bladder tissues were harvested and fixed in NBF to evaluate possible toxicity of freeze-dried blueberries for these tissues.

2.3.5. Experimental design: DNA adduct study
The effect of 10% freeze-dried blueberries on NMBA-induced formation of O\(^6\)-methylguanine (O\(^6\)-meGua) adducts in the rat esophagus was determined in a second short-term bioassay. The design of the adduct experiment is given in Table 2.2. and Figure 2.2. Three groups of 15 rats were randomized and placed either on AIN-76A diet or AIN-76A diet containing 10% lyophilized blueberries for a period of two. A single s.c. injection of NMBA at 0.5 mg/kg body weight was administered to all rats 2 weeks after the initiation of control or 10% blueberry diets. Twenty-four hours after NMBA treatment, the rats were euthanized by CO\(_2\) asphyxiation, and the esophagus of each animal excised, split longitudinally and the epithelium stripped from the underlying mucosa, frozen immediately in liquid nitrogen, and stored at -80°C. DNA was isolated from 3 pooled mucosal samples (five total samples per group), subjected to acid hydrolysis, and the levels of O\(^6\)-meGua quantified using strong cation exchange HPLC as described (19, 34).

2.3.6. Statistical analysis

All statistical procedures were carried out using the NCSS 97 statistical software package (NCSS Statistical Software, Kaysville, UT). Body weight, food consumption, tumor multiplicity and tumor size data were analyzed for statistical significance (\(P < 0.05\)) using analysis of variance (ANOVA) followed by Neuman-Keuls’ multiple comparisons and Kruskal-Wallis tests. Differences in tumor incidence were determined by Fisher’s exact probability test and \(\chi^2\) and Kruskal-Wallis tests. DNA adduct levels were analyzed by linear regression an ANOVA in order to detect differences between means and to calculate SE’s.
### Table 2.1: Experimental design for freeze-dried blueberry anti-initiation bioassay.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. rats</th>
<th>Amount Admin. (ml)</th>
<th>NMBA Dose Admin. (mg/kg b.w.)</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMSO + H₂O</td>
<td>10</td>
<td>0.2</td>
<td>0</td>
<td>Control AIN-76A</td>
</tr>
<tr>
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<td>None</td>
<td>10</td>
<td>0.2</td>
<td>0</td>
<td>AIN-76A + 10% BB</td>
</tr>
<tr>
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<td>NMBA</td>
<td>25</td>
<td>0.2</td>
<td>0.25</td>
<td>Control AIN-76A</td>
</tr>
<tr>
<td>4</td>
<td>NMBA</td>
<td>25</td>
<td>0.2</td>
<td>0.25</td>
<td>AIN-76A + 5% BB</td>
</tr>
<tr>
<td>5</td>
<td>NMBA</td>
<td>25</td>
<td>0.2</td>
<td>0.25</td>
<td>AIN-76A + 10% BB</td>
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</tbody>
</table>

Abbreviations: NMBA, *N*-nitrosomethylbenzylamine; BW, body weight; BB, freeze-dried blueberries.

### Table 2.2: Experimental design for DNA adduct study.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. rats</th>
<th>Amount Admin. (ml)</th>
<th>NMBA Dose Admin. (mg/kg b.w.)</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMSO + H₂O</td>
<td>15</td>
<td>0.2</td>
<td>0</td>
<td>Control AIN-76A</td>
</tr>
<tr>
<td>2</td>
<td>NMBA</td>
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<td>0.2</td>
<td>0.5</td>
<td>Control AIN-76A</td>
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<tr>
<td>3</td>
<td>NMBA</td>
<td>15</td>
<td>0.2</td>
<td>0.5</td>
<td>AIN-76A + 10% BB</td>
</tr>
</tbody>
</table>

Abbreviations: NMBA, *N*-nitrosomethylbenzylamine; BW, body weight; BB, freeze-dried blueberries.

Table 2.1: Experimental design for freeze-dried blueberry anti-initiation bioassay.

Table 2.2: Experimental design for DNA adduct study.
Figure 2.1. Experimental protocol for the anti-initiation bioassay. Rats were treated with NMBA at 0.25 mg/kg body weight once a week for 15 weeks. Freeze-dried blueberries were administered 2 weeks prior to initiation of NMBA treatment and for the duration of the study.

Figure 2.2. Experimental protocol for the DNA adduct study. Rats were treated with one dose of 0.5 mg/kg body weight at the end of day 14. Twenty-four hours later (day 15), the animals were harvested. Freeze-dried blueberries were administered for the entire of study.
2.4. Results

2.4.1. Results from anti-initiation bioassay

There were no significant differences in mean body weight or food consumption for the duration of the bioassay (Figure 2.3 and 2.4). Histopathological examination of sections of the liver, colon, stomach, kidney, spleen, heart, and bladder tissues from animals fed 10% berries indicated that the berries did not elicit toxic effects in any of these organs. The effects of freeze-dried blueberries on NMBA-induced tumorigenesis in the rat esophagus are shown in Table 2.3. There was no significant difference in either tumor incidence, size or multiplicity in berry fed animals (Groups 4 and 5) versus animals treated with NMBA only (Group 3).

2.4.2. Results from DNA adduct study

Metabolic activation of NMBA in the rat esophagus results in DNA methylation at the $N^7$ and $O^6$ positions of guanine (35). While the $N^7$ adduct occurs at higher levels than the $O^6$ adduct, $O^6$-meGua formation leads to single nucleotide G->A transition mutations that are associated with carcinogenic effects (36). A study was performed therefore, to determine whether freeze-dried blueberries fed at a dose of 10% of the diet would inhibit NMBA-induced $O^6$-MeGua adduct formation in the rat esophagus. Analysis of the data shows that there was no significant difference in the levels of $O^6$-meGua in the esophagi of the NMBA treated group verses animals treated with NMBA + 10% blueberries (Table 2.4).
Figure 2.3. Body weight data for freeze-dried blueberries anti-initiation bioassay. Group symbols are as follows: vehicle control (♦); 10% freeze-dried blueberries (●); NMBA control (▲); NMBA + 5% freeze-dried blueberries (×); NMBA + 10% freeze-dried blueberries (★).

Figure 2.4. Food consumption data for freeze-dried blueberries anti-initiation bioassay. Group symbols are as follows: vehicle control (♦); 10% freeze-dried blueberries (●); NMBA control (▲); NMBA + 5% freeze-dried blueberries (×); NMBA + 10% freeze-dried blueberries (★).
<table>
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<tr>
<th>Group</th>
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<th>No. rats</th>
<th>Tumor incidence (%)</th>
<th>Tumors Multiplicity (mean ± SE)</th>
<th>Tumor Size mm³ (mean ± SE)</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>Vehicle</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>10% BB</td>
<td>10</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>3</td>
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<td>24</td>
<td>91.3</td>
<td>2.2 ± 0.29</td>
<td>5.9 ± 7.9</td>
</tr>
<tr>
<td>4</td>
<td>NMBA + 5% BB</td>
<td>24</td>
<td>91.7</td>
<td>2.5 ± 0.29</td>
<td>5.5 ± 12.7</td>
</tr>
<tr>
<td>5</td>
<td>NMBA + 10% BB</td>
<td>23</td>
<td>95.7</td>
<td>2.6 ± 0.28</td>
<td>3.9 ± 4.9</td>
</tr>
</tbody>
</table>

Abbreviations: NMBA, N-nitrosomethylbenzylamine; BW, body weight; BB, freeze-dried blueberries.

Table 2.3: Effect of freeze-dried blueberries on NMBA-induced tumorigenesis in the rat esophagus.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>pmol O⁶-MeGua/mg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMSO + H₂O</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>NMBA</td>
<td>3.3 ± 1.1</td>
</tr>
<tr>
<td>3</td>
<td>NMBA + 10% BB</td>
<td>2.9 ± 0.6</td>
</tr>
</tbody>
</table>

Abbreviations: NMBA, N-nitrosomethylbenzylamine; BW, body weight; BB, freeze-dried blueberries.

Table 2.4: Effect of freeze-dried blueberries on formation of O⁶-methylguanine in the esophagus of rats treated with NMBA.
2.5. Discussion

A 25-week carcinogenesis bioassay was conducted to evaluate the ability of freeze-dried blueberries to inhibit NMBA tumorigenesis in the rat esophagus. At 5 and 10% of the diet, the blueberries were found to be ineffective in reducing both the incidence and size of NMBA-induced tumors in the esophagus. In addition, tumor multiplicity data collected during sacrifice showed similar results. These results are consistent with the observation that the blueberries did not reduce the level of formation of O\(^6\)-meGua adducts in esophageal DNA. Unlike strawberries and black raspberries therefore, blueberries appear to lack compounds that influence the metabolic activation and/or detoxification of NMBA leading to decreased levels of DNA damage. As mentioned previously, our laboratory has shown that feeding rats freeze-dried strawberries and black raspberries significantly reduced esophageal tumors. Furthermore, both berry types reduced the levels of O\(^6\)-meGua adducts. In addition, it has been shown that the naturally occurring polyphenol, ellagic acid, inhibits the formation of O\(^6\)-meGua adducts from NMBA in the rat esophagus (37, 38). Unlike strawberries and black raspberries, blueberries contain only a small amount of ellagic acid. Thus, the lack of an inhibitory effect of blueberries on O\(^6\)-meGua formation in the rat esophagus might be explained, at least in part, by their relatively low amounts of ellagic acid. \textit{In vitro} studies using blueberry extracts showed similar results. This is not the entire explanation however, since we have shown that the inhibitory effect of freeze-dried strawberries and black raspberries on NMBA
metabolism and tumorigenesis in the rat esophagus is not due to ellagic acid alone (17-19). Nutrient analyses indicate that blueberries contain lower amounts of phenolics such as ferulic and p-coumeric acid in addition to ellagic acid. It may be plausible that ellagic acid in combination with one or more phenolic compounds is responsible for the berries inhibitory activity. It may also be possible that the anthocyanins may exhibit an antioxidant effect similar to that observed in the beta-carotene study. Another explanation for blueberries inability to inhibit tumors may be the source of blueberries obtained. Studies by Kalt and al. show that low bush blueberries were consistently higher in anthocyanins, total phenolics, and antioxidant capacity, compared to high bush blueberries (25).

Unlike strawberries and black raspberries, our data suggests that freeze-dried blueberries, at dietary concentrations of 5 and 10%, were ineffective at reducing the incidence and size of NMBA-induced tumors in the rat esophagus. Thus, blueberries are not an effective chemopreventive against NMBA-induced esophageal tumors in an anti-initiation bioassay. One explanation for this is that the blueberries did not reduce levels of NMBA-induced DNA adduct formation in the esophagus. This may be due, in part, to their relatively low level of ellagic acid and/or a combination of other compounds (39). This conclusion may not be the only explanation why the blueberries were not effective since previous studies have shown that the inhibitory effects of black raspberries and strawberries were not due to ellagic acid alone (18, 19).
2.6. References


CHAPTER 3

EVALUATION OF DIFLUOROMETHYLORNITHINE (DFMO) ON POST-INITIATION EVENTS OF NMBA-INDUCED TUMORIGENESIS IN THE RAT ESOPHAGUS

3.1. Abstract

The rat esophagus is a useful model for human esophageal squamous cell carcinoma. Tumors can easily be induced in the rat esophagus by treatment of the rats with the nitrosamine carcinogen, \( N \)-nitrosomethylbenzylamine (NMBA). NMBA induces esophageal tumors in the rat when administered once weekly for 15 weeks or 3 times per week for 5 weeks at a dose of 0.25 mg/kg/injection. Also, NMBA is a selective carcinogen inducing tumors only in specific tissues such as the esophagus and nasal cavity. NMBA metabolism involves a series of reactions which eventually lead to the formation of an unstable carbonium ion that can directly affect DNA by methylating the O\(^6\) and/or N-7 positions of guanine.

DFMO is an enzyme-activated irreversible inhibitor of ornithine decarboxylase, the rate-limiting enzyme in polyamine synthesis, and decreases intracellular levels of putrescine and spermidine in the skin and other vital tissues. In conjunction with the administration of model carcinogens, DFMO
significantly reduced tumor incidence in several mammalian in vivo tests for chemopreventive activity. Past studies have shown DFMO to be an effective inhibitor of N-nitrosomethylbenzylamine (NMBA)-induced tumorigenesis in the rat esophagus when administered before, during and after carcinogen treatment. The main purpose of this study was to determine whether DFMO is associated with a significant reduction in rat esophageal tumorigenesis when administered in the diet following pre-initiation of animals with NMBA.

3.2. Introduction

Esophageal cancer ranks eighth in cancer incidence worldwide and is the fifth most common cause of cancer death (1). Current data estimates approximately 13,000 deaths from esophageal cancer in the United States in 2003, while it is estimated that 13,900 new cases will occur in 2004 (2). There are many etiologic factors associated with the pathogenesis of the disease including tobacco and alcohol use, exposure to nitroso compounds found in the food, consumption of moldy foods and hot beverages, vitamin and mineral deficiencies, exposure to nitrosamines (found in the food, environment, and water) and hereditary factors (3). The F344 rat has been the most extensively used model for human esophageal SCC (4). The multistage progress of tumorigenesis in the rat esophagus closely mimics the various stages of premalignant and malignant progression of human esophageal SCC observed in humans (4, 5,).
In addition to taking a food-based approach, our laboratory has been actively examining pure compounds for their ability to inhibit tumorigenesis in the rat esophagus. Studies conducted by Stoner et. al have shown many agents such as polyphenols, isothiocyanates, S,S’-1,4-phenylene-bis (1,2-ethanediyl) bis-isothiourea (PBIT) and L-708 to be effective chemopreventives to inhibit NMBA-induced tumorigenesis. DFMO, in particular, is especially interesting to examine because studies by Fong et. al have shown that it is highly effective chemopreventive agent in esophageal tumorigenesis in zinc-deficient rats (7). Before discussing the importance of DFMO as a potential chemopreventive, a discussion of its role in polyamine biosynthesis is given.

*In vivo* and clinical trials indicate that the levels of polyamines are elevated in a number of precancerous and cancerous lesions (8, 9). Polyamines are defined as a small and relatively discrete class of biological substances that consist of aliphatic nonprotein nitrogenous bases found in a number of different cells and tissues (10). The actual function of these compounds is not entirely clear but studies have shown that they are necessary for normal cellular growth and differentiation (8, 11). Polyamines are growth factors in both eucaryotic and procaryotic cells and are synthesized in highly regulated pathways within these cells. They possess a low molecular weight and are positively charged at physiological pH. Due to their positive charge, polyamines have a tendency to bind to negatively charged molecules such as nucleic acids, acidic phospholipids and various types of proteins (12). They also have the ability to bind to DNA. Some of the early known polyamines that were discovered and characterized by
the 1930's include putrescine, cadaverine, spermidine, and spermine (see Introduction – Chapter 1 under Single agent approach – Table 1.6 and Figure 1.6). All of these polyamines originated from the compound ornithine. Putrescine and cadaverine are primary diamines and are known products of bacterial metabolism (9). Spermidine and spermine are a triamine and tetramine, respectively, that contain primary and secondary amines. In general, spermidine and spermine are present in millimolar concentrations in cells, while putrescine levels are somewhat lower. Although each serves an important function, spermidine, spermine, and their precursor putrescine are all involved in polyamine biosynthesis. An absence of polyamines in the body will cause cells to cease to grow and proliferate but not necessarily die (9). External stimuli may increase intracellular polyamine concentrations thus leading to rapid cell proliferation in both normal and neoplastic cells and tissues. In addition, polyamines have an important role in the regulation of some major classes of cation channels (8). For example, spermidine is a precursor of an unusual amino acid hypusine needed for the synthesis of translation initiation factor 5A (12).

ODC is the enzyme responsible for converting ornithine to putrescine, the first step in polyamine biosynthesis (9). High levels of ODC and ornithine have been observed in a number of tumors and proliferating cells (13-19). Elevated levels of ODC expression may result in rapid cellular proliferation. Thus, a viable means of chemoprevention may be targeting polyamine biosynthesis. An effective means at inhibiting polyamine biosynthesis can be achieved by one of
two ways: (a) using an agent that can compete with ornithine and / or (b) using an agent and / or agents that can compete with ODC (17).

Difluoromethylornithine (DFMO) is an irreversible inhibitor of ODC thus depleting the levels of cellular putrescine and spermidine and having a limited effect on spermine levels (20). DFMO has been shown to exhibit to exhibit strong antiproliferative activity against many tumors. Furthermore, DFMO is effective at inhibiting tumorigenesis in specific organs of animals such as the bladder, colon, mammary gland, liver, stomach, skin, and esophagus (20). Our rationale for using this agent comes from data which indicates mRNA levels for ODC in human esophageal SCC are $14.6 \pm 3.7$ times higher than that in normal paired tissues (15, 21). In addition, studies by Kelloff et. al found higher ODC activity in the esophagus when compared to other tissues such as the stomach, colon, and liver (22). In a short-term study in our laboratory, preliminary data showed that DFMO is an effective inhibitor of $N$-nitrosomethylbenzylamine (NMBA)-induced tumorigenesis in the rat esophagus when administered before, during and after carcinogen treatment in a dose-dependent manner. Furthermore, DFMO produced a significant, dose dependent reduction in tumor incidence and multiplicity. At 25 weeks, 500 and 1,000 ppm DFMO significantly reduced tumor incidence by 24 and 43% and tumor multiplicity by 41 and 77%, respectively. Thus, DFMO was an effective chemopreventive when given in an anti-initiation scheme. We wanted to determine, however, if DFMO has any effect on the promotion/progression stages of NMBA-induced esophageal tumorigenesis. The major purpose of this study, therefore, was to determine
whether DFMO, at a dose of 1,000 ppm is associated with a significant reduction in rat esophageal tumorigenesis when administered in the diet following pre-initiation of animals with NMBA. The effect on DFMO on pre-neoplastic lesion formation was also evaluated.

3.3. Materials and Methods

3.3.1 Animals

Male F-344 rats, 5-6 weeks-old, were purchased from Harlan Sprague-Dawley (Indianapolis, IN). The animals were housed 3 per cage under standard conditions (20 ± 2°C; 50 ± 10% relative humidity; 12-hour light/dark cycles). Rats were maintained on modified AIN-76A diet containing 20% casein, 0.3% D,L-methionine, 52% cornstarch, 13% dextrose, 5% cellulose, 5% corn oil, 1% AIN vitamin mixture, 3.5% AIN salt mixture and 0.2% choline bitartrate (Dyets, Bethlehem, PA). Food and water were provided ad libitum throughout the bioassay. Hygienic conditions were maintained by twice weekly cage changes. Body weight and food consumption measurements were recorded weekly for the duration of the study.

3.3.2. Chemicals

N-Nitrosomethylbenzylamine (NMBA), obtained from Ash Stevens, Inc. (Detroit, MI), was determined to be greater than 98% pure by HPLC analysis. Dimethyl sulfoxide (DMSO) was purchased from Sigma Chemical Company (St. Louis, MO). DFMO was obtained from The National Cancer Institute Chemical Repository. On a weekly basis, a Hobart mixer was used to mix 1,000 ppm of DFMO into modified AIN-76A purified diet.
3.3.3. Diet preparation

Rats were maintained on a modified AIN-76A purified diet containing 20% casein, 0.3% D, L-methionine, 52% cornstarch, 13% dextrose, 5% cellulose, 5% corn oil, 3.5% AIN salt mixture, 1% AIN vitamin mixture and 0.2% choline bitartrate (Dyets, Bethlehem, PA). Diet containing DFMO was prepared on a weekly basis and kept at 4°C. For the present study, 1,000 ppm of DFMO was added directly to modified AIN-76A purified diet and mixed for 20 minutes in a Hobart mixer. On a weekly basis, portions of DFMO diets and control diet were placed into glass feeding jars and fed to the animals.

3.3.4. Experimental Design: Post-initiation biosasay

To evaluate the effect of difluoromethylornithine (DFMO) on NMBA-induced tumorigenesis in the rat esophagus, animals were randomized into 4 groups and fed a modified AIN-76A purified diet (Table 3.1). Rats were then injected subcutaneously with NMBA (0.25 mg/kg body weight) three times a week for five weeks (Figure 3.1). Following the final NMBA treatment, animals remained on AIN-76A diet or were given AIN-76A diet containing 1,000 ppm of DFMO for the duration of the bioassay (25 weeks). At week 25, the rats were euthanized by CO₂ asphyxiation. The esophagus of each animal was opened longitudinally and the surface tumors were mapped, counted and measured. Lesions greater than 0.5 mm in diameter were considered to be tumors. Assuming a spheroid shape, a volume estimate for each papilloma was calculated (23). The esophagi were then fixed in 10% neutral buffered formalin (NBF) and processed for histopathology. Multiple H&E stained slides were
prepared from the esophagi of five rats per group and used for the quantification of preneoplastic lesions (i.e., hyperplasia, simple leukoplakia and dysplastic leukoplakia). Although a preliminary toxicity study ruled this out, sections of the liver, colon, stomach, kidney, spleen, heart, and bladder tissues were harvested once again and fixed in NBF to evaluate for possible toxicity of DFMO.

3.3.5. Statistical analysis

All statistical procedures were carried out using the NCSS 97 statistical software package (NCSS Statistical Software, Kaysville, UT). Body weight, food consumption, tumor multiplicity and tumor size data were analyzed for statistical significance \( (P < 0.05) \) using analysis of variance (ANOVA) followed by Neuman-Keuls’ multiple comparisons and Kruskal-Wallis tests. Differences in tumor incidence were determined by Fisher’s exact probability test and \( \chi^2 \) and Kruskal-Wallis tests.

3.3.6. Histological grading of preneoplastic lesions

The esophagi from 10 animals per group were cut into thirds and paraffin embedded on edge. Sections of the esophagi were cut into 4-\( \mu \)m sections and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) where they were sectioned and stained with hematoxylin and eosin. The entire esophagus was then scanned at 100-X magnification. Each viewing field was categorized into one of five histological categories: normal epithelium, epithelial hyperplasia, low-grade dysplasia, and high-grade dysplasia. Normal epithelium was described as having the following: a stratified squamous epithelium, a basal layer consisting of two to five cell rows of spinous cells; and having a nuclear:
cytoplasmic ratio of 1:4. Epithelial hyperplasia was described as the following: thickened epithelium with 3 to 7 rows of basal cells and a slight increase in the keratin layer. Low-grade dysplasia was described as highly progressed hyperplasia accompanied by cellular disorganization, nuclear atypia and a further increase in size of the keratin layer (whitish in color). High-grade dysplasia displayed disorderly cellular organization with more pronounced nuclear atypia (Figure 1.4: Classification of histopathological and morphogenetic changes that occur during NMBA-induced esophageal tumorigenesis in the F344 rat). The classification scheme used was a modification of criteria developed by Pozharisski et al., with consideration of the gross and microscopic descriptions of hyperplasia and dysplasia given in Cotran et al. (24).
<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. rats</th>
<th>Amount Admin. (ml)</th>
<th>NMBA Dose Admin. (mg/kg b.w.)</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMSO + H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>6</td>
<td>0.2</td>
<td>0</td>
<td>Control AIN-76A</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>6</td>
<td>0.2</td>
<td>0</td>
<td>AIN-76A + DFMO, 1,000 ppm</td>
</tr>
<tr>
<td>3</td>
<td>NMBA</td>
<td>31</td>
<td>0.2</td>
<td>0.25</td>
<td>Control AIN-76A</td>
</tr>
<tr>
<td>4</td>
<td>NMBA</td>
<td>31</td>
<td>0.2</td>
<td>0.25</td>
<td>AIN-76A + DFMO, 1,000 ppm</td>
</tr>
</tbody>
</table>

Abbreviations: NMBA, N-nitrosomethylbenzylamine; BW, body weight; DFMO, difluoromethylornithine.

Table 3.1: Experimental design for the DFMO post-initiation bioassay.
Figure 3.1: Experimental protocol for the DFMO post-initiation bioassay. Rats were treated with NMBA at 0.25 mg/kg body weight three times a week for five weeks. DFMO was administered in the diet at 1,000 ppm after NMBA treatment and for the duration of the 25-week bioassay.
3.4. Results

There were no significant differences in mean body weight or food consumption for the duration of the bioassay (Figures 3.2 and 3.3). Histopathological examination of sections of the liver, colon, stomach, kidney, spleen, heart, and bladder tissues from animals fed 1000 ppm of DFMO indicated that the drug did not elicit toxic effects in any of these organs. DFMO reduced tumor multiplicity by 40% when given after carcinogen administration (Table 3.2; 1.53 to 0.94 tumors/rat). As shown in Table 3.3 there was no difference in preneoplastic lesions between the NMBA and NMBA plus DFMO treated groups. Histologic data revealed no difference in preneoplastic lesion formation in rats treated with NMBA versus those treated with NMBA + DFMO (Table 3.2). Tumor data, however, suggests that DFMO may be an effective agent against NMBA-induced esophageal tumorigenesis when administered in the diet post-initiation. As shown in Table 3.3, tumor multiplicity decreased from an average of 1.53 tumors/group in the NMBA-treated groups to an average of 0.94 tumors/group in the NMBA + 1,000 ppm of DFMO.
Figure 3.2: Effect of DFMO on mean body weight in a post-initiation bioassay. Body weight was measured weekly and graphed over time.

Figure 3.3: Effect of DFMO on food consumption in a post-initiation bioassay. Food consumption was measured weekly and graphed over time.
<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. rats</th>
<th>Normal</th>
<th>Epithelial hyperplasia</th>
<th>Low-grade dysplasia</th>
<th>High-grade dysplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMSO+ H₂O</td>
<td>6</td>
<td>115 (76.7)</td>
<td>31 (20.7)</td>
<td>4 (2.7)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>2</td>
<td>DFMO, 1000 ppm</td>
<td>6</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>3</td>
<td>NMBA</td>
<td>31</td>
<td>84 (16.5)</td>
<td>201 (39.6)</td>
<td>161 (31.7)</td>
<td>62 (12.2)</td>
</tr>
<tr>
<td>4</td>
<td>NMBA+ DFMO 1000 ppm</td>
<td>31</td>
<td>60 (12.1)</td>
<td>215 (43.3)</td>
<td>141 (28.4)</td>
<td>80 (16.1)</td>
</tr>
</tbody>
</table>

Abbreviations: NMBA, N-nitrosomethylbenzylamine; BW, body weight; DFMO, difluoromethylornithine.

Table 3.2: Effect of difluoromethylornithine on NMBA-induced preneoplastic esophageal lesions in the F-344 rat.
<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. rats</th>
<th>Tumor incidence (%)</th>
<th>Tumor Multiplicity (mean ± SE)</th>
<th>Tumor Size (mm³) (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMSO + H₂O</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>DFMO, 1000 ppm</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>NMBA</td>
<td>31</td>
<td>77.42</td>
<td>1.53 (0.24)</td>
<td>5.05 ± 0.86</td>
</tr>
<tr>
<td>4</td>
<td>NMBA + DFMO, 1000 ppm</td>
<td>31</td>
<td>58.06</td>
<td>0.94(0.18)²</td>
<td>4.71 ± 2.69</td>
</tr>
</tbody>
</table>

Abbreviations: NMBA, N-nitrosomethylbenzylamine; BW, body weight; DFMO, difluoromethylornithine.

² The p value is calculated to be p = 0.056

Table 3.3: Anti-promotion / progression effects of difluoromethylornithine NMBA-induced esophageal tumorigenesis in the F344 rat.
3.5. Discussion

In the present study, groups of 31 male F-344 rats were injected subcutaneously with NMBA (0.25 mg/kg body weight) 3 times per week for 5 weeks. Seventy-two hours following the final NMBA treatment, and for the duration of the study, DFMO was administered in the diet at 1,000 ppm. Twenty-five weeks following the initiation of NMBA treatment, DFMO reduced tumor multiplicity by 40% (p = 0.056). These data suggest that DFMO is a potentially useful chemopreventive agent in the rat esophagus. Additional studies are needed to determine whether tumor reduction is correlated with ODC activity and polyamine levels. In addition, synergism studies involving DFMO at multiple doses, coupled with food-based chemopreventives (such as berries) would be useful to evaluate potential dose-responsive inhibitory effects from the combination of these agents.

A 25-week post-initiation bioassay was conducted to evaluate the ability of DFMO to inhibit NMBA tumorigenesis in the rat esophagus. At a dose of 1000 ppm, DFMO was found to be effective in reducing tumor multiplicity by 40%. Tumor size was not affected by the DFMO treatment due to the high degree of variation in tumor size among the groups used in the study. These results are consistent with our previous observation that DFMO is effective at reducing tumors when given before during and after NMBA treatment. Therefore, DFMO at the dose of 1,000 ppm is effective when given in the diet before, during and after NMBA treatment, and also when administered only after NMBA exposure.
Thus, DFMO exhibits both anti-initiation and anti-promotion/progression effects. Many studies have reported a correlation between ODC expression and polyamine levels in tumor promotion stages of various cancers. DFMO acts by competitively binding to ODC thus reducing the conversion of ornithine to putrescine, spermidine and spermine. Unlike other ODC inhibitors, DFMO structurally resembles ornithine (20, 22). When DFMO comes into contact with ODC, it effectively competes with the enzyme active site (20, 22). This, in turn, reduces overall levels of putrescine, spermidine, and spermine. Although DFMO may initially act to increase levels of polyamines, this increase eventually levels off to a net decrease. Quantification of polyamines in tissues by hplc has confirmed this (25). When tissues from human esophageal samples were taken immediately after biopsy, levels of polyamines were shown to decrease with administration of DFMO (21). Another point concerning DFMO is that it is very specific and irreversible, unlike other ODC inhibitors which are non-specific and reversible.

In this study, we have shown that DFMO appears to be an effective inhibitor of rat esophageal tumorigenesis when added into the diet at 1,000 ppm and following treatment of rats with NMBA. Although this was a preliminary study with a borderline significant value (p = 0.056), additional studies are underway to confirm whether DFMO is, in fact, an effective inhibitor against NMBA-induced tumorigenesis. Combination strategies using DFMO with other esophageal inhibitors, such as freeze-dried berry preparations, may be useful to examine in the future. Our laboratory has shown that freeze-dried berry preparations, in
particular strawberries and black raspberries, are effective at reducing esophageal tumor occurrence in NMBA-treated rats (23, 26). Black raspberries were also effective at reducing esophageal cell proliferation in NMBA-treated rats and colon adenocarcinomas in azoxymethane-induced colon tumors in rats (26, 27).
3.6. References


CHAPTER 4

THE THERAPEUTIC EFFECT OF FREEZE-DRIED BLACK RASPBERRIES ON N-NITROSOETHYLBNZYLAMINE-INDUCED TUMORIGENESIS IN THE F344 RAT ESOPHAGUS

4.1. Abstract

Epidemiological data suggest an inverse relationship between consumption of fruit and vegetables and the occurrence of several types of cancer. In particular, the incidence of squamous cell carcinoma of the human esophagus has been linked to diets deficient in fruit and vegetables. Recently, we have taken a “food based” approach to the prevention of esophageal squamous cell carcinoma in rodents. We found that the administration of freeze-dried strawberries and black raspberries, at concentrations of 5 and 10% in a synthetic diet, produced significant decreases in N-nitrosomethylbenzylamine (NMBA)-induced esophageal tumors in rats. The berries were effective when administered in the diet before, during and after fifteen weeks of NMBA treatment, and also when given continuously in the diet beginning one week after five weeks of NMBA treatment. Thus, the berries exhibited both anti-initiation and anti-promotion/progression effects. In addition, organic extracts from strawberries and black raspberries were shown to inhibit benzo[a]pyrene-induced transformation of Syrian hamster embryo (SHE) cells in vitro and to reduce AP-1,
NFκB and VEGF expression in CD41 mouse epidermal cells. In the present study, we determined whether berries might exhibit therapeutic effects against esophageal cancer in rats. Six week-old male F344 rats were placed on AIN-76A diet and injected s.c. with 0.5 mg/kg NMBA once per week for 15 weeks. Four weeks later (at 19 weeks), when they had an average of 5 to 6 papillomas per esophagus, NMBA-treated rats were started on diets containing either 0, 5, 10, or 20% freeze-dried black raspberries. For several weeks after initiation of berry treatment, the survival of rats in berry-fed groups was higher than in rats fed AIN-76A control diet. After seven weeks of berry treatment, all surviving rats were sacrificed (at 26 weeks) and esophageal tumor incidence, multiplicity and size determined. In animals fed 10% and 20% black raspberries, there were no significant differences in tumor incidence, multiplicity or size when compared to NMBA-treated controls. Animals fed 5% black raspberries, however, had a significant reduction in tumor multiplicity when compared to NMBA-treated controls. These results suggest that dietary administration of freeze-dried black raspberries may have some therapeutic value, and that the higher concentrations (10 and 20%) of berries may be less effective than the lower (5%) concentration. Similar results were observed in post-initiation bioassays in which 5% black raspberries were found to be more effective in preventing NMBA-induced esophageal tumorigenesis than 10% black raspberries. Additional bioassays are underway to further assess the therapeutic effect of berries on esophageal tumorigenesis.

4.2. Introduction
Worldwide, esophageal cancer ranks eighth in cancer incidence and is the fifth most common cause of cancer death (1). Current data estimates approximately 13,000 deaths from esophageal cancer in 2003, while it is estimated that 12,300 new cases will occur in 2003 (2, 3). In the United States, esophageal cancer is relatively uncommon, but it is associated with a 5-year survival rate of only 12% from the disease at all stages while 75% die within a year of diagnosis (4-6). Moreover, mortality estimates represented 95% of the expected incidence in 2003 (1). Regions of the world that have the highest incidence rates include China and other Asian countries, the Transkei region of South Africa, Iran, France, the Caribbean and Puerto Rico (6, 7). Esophageal cancer is of two major types; squamous cell carcinoma (SCC) which occurs principally in the middle and distal portion of the esophagus and adenocarcinoma which arises at the distal portion of the esophagus. Approximately 90% of all esophageal cancers worldwide are squamous cell carcinomas (8-10). Adenocarcinomas, which arise from a preneoplastic lesion termed Barrett's esophagus, account for 5% of esophageal malignancies and their rate of occurrence is increasing rapidly in the United States. The other 5% of esophageal cancers are mesenchymal tumors (11).

Among the risk factors positively associated with the development of SCC of the esophagus include individual and combined use of tobacco and alcohol consumption, high intakes of pickled and moldy foods, consumption of hot and spicy foods and beverages, ingestion of nitrosamines and benzo(a)pyrene, the presence of esophagitis, dietary deficiencies in certain vitamins and minerals,
and low intake of fruits and vegetables (12-15). Some of these products are frequently contaminated with N-nitrosamine carcinogens and/or fungal toxins. Extensive research in China and South Africa has suggested that N-nitroso compounds, including N-nitrosomethylbenzylamine (NMBA), and their precursors are probable etiological factors for esophageal SCC in these high incidence areas (16). In view of these exposures, one approach to the prevention of esophageal SCC is through changes in lifestyle, including avoidance of tobacco and alcohol, especially in combination, and an increased consumption of vitamins and minerals (found primarily in fruits and vegetables) in the diet (17, 18). Additional benefits may be realized with the elimination of high salt-pickled foods that may be contaminated with microbial toxins, nitrosamines and their precursors and reduction in the intake of hot beverages (19, 20). Chemoprevention, to address factors associated with the etiology and progression of the disease, is another viable approach (21-23).

The F344 rat esophageal model is a useful model to study NMBA-induced tumorigenesis, histopathological changes observed in the esophagus, and evaluation of multiple chemopreventive agents (24). NMBA is a selective carcinogen inducing tumors only in specific tissues such as the esophagus and nasal cavity (24-25). NMBA induces esophageal tumors in the rat when administered once weekly for 15 weeks or 3 times/week for 5 weeks at a dose of either 0.25 or 0.5 mg/kg body weight (24). NMBA metabolism involves a series of reactions which eventually lead to the formation of an unstable carbonium ion that can directly affect DNA by methylating the O⁶ and N⁷ positions of guanine.
(24-26). Currently, clinical trials are identifying some chemopreventive agents that appear to be useful for reducing the risk for development of esophageal SCC in humans (14).

Although primary chemoprevention is a viable approach to preventing disease or having an effect on either the initiation and/or promotion/progression states of carcinogenesis, it is of little use in the majority of individuals with esophageal SCC who are at the advanced metastatic stages of the disease at the time of diagnosis (27, 28). Although, a radical operation which includes removal of the esophagus, endoscopic resection, improves survival in patients with advanced stages of esophageal SCC, more than half of the patients return with local recurrence or distant metastases (27). Esophageal cancer can be treated at earlier stages using mass screening detection and adjuvant chemotherapy and/or radiotherapy. These treatment options, however, yield similar results as those seen with patients undergoing surgery. Therefore, the use of chemoprevention to treat precancerous lesion such as in situ dysplasia before the disease transforms into the irreversible malignant state is another approach (22, 23). There is little evidence, however, of the benefits of using chemopreventives to treat tumors that have already developed.

Epidemiological data suggest an inverse relationship between consumption of fruit and vegetables and the occurrence of several types of cancer (29, 30). In particular, the incidence of SCC of the esophagus has been linked to diets deficient in fruit and vegetables (31-36). By using a food-based approach to cancer chemoprevention, our laboratory has evaluated freeze-dried
berries in different carcinogen-induced tumor models including the esophagus, colon, lung, and oral cavity (37). Among the berries, black raspberries are of special interest due to their high content of multiple chemopreventives including vitamins A, C, and E, folic acid, calcium, selenium, beta-sitosterol, ellagic and ferulic acids, and multiple anthocyanins as outlined in Table 1.5 in the Introduction (38, 39). Kresty et. al, found that the administration of freeze-dried black raspberries, at specific concentrations in a synthetic diet, produced significant decreases in NMBA-induced esophageal tumors in rats (40). Specifically, 5 and 10% dietary freeze-dried black raspberries produced a 39% and 49% reduction, respectively, in esophageal tumor multiplicity when administered in the diet before, during and after treatment of rats with NMBA. When administered only after carcinogen treatment, 5 and 10% dietary freeze-dried black raspberries produced a 37% and 31% reduction in tumors, respectively (40). Therefore, the black raspberries were effective when given in the diet before, during and after NMBA treatment, and also when administered only after NMBA exposure. Black raspberries were also found to reduce preoplastic lesion formation and cell proliferation rates (40). Kresty et. al also showed that 5 and 10% dietary black raspberries inhibit the formation of the promutagenic adduct, O\textsuperscript{6}-methylguanine (O\textsuperscript{6}-meGua), by 73 and 80% respectively, after a single dose of NMBA at 0.25 mg/kg. Further studies revealed that when the dose of NMBA was increased to 0.50 mg/kg, 5% black raspberries significantly inhibited adduct formation by 64% (40). Although it has been speculated that the chemopreventive ellagic acid is responsible for the
inhibitory effect of berries, this is not likely to be the case (37, 41, 42). Black raspberries were also found to be very effective in inhibiting azoxymethane (AOM)-induced carcinogenesis in the rat colon when administered in the diet post-initiation (43). Oxidative stress, as shown by reduced levels of urinary 8-OH-deoxyguanosine in the urine of AOM-treated animals, was also reduced by black raspberries (43). Studies by Casto et. al, have shown dietary administration of black raspberries are effective at inhibiting tumor formation in the oral cavity in the hamster cheek pouch (44). In vitro studies with black raspberries have yielded similar results as the in vivo studies. Extracts isolated from black raspberries inhibit benzo(a)pyrene-induced transformation of Syrian hamster embryo (SHE) cells in vitro (45). In addition, extracts from black raspberries were shown to be effective at reducing AP-1, NFκB and VEGF expression in CD41 mouse epidermal cells (46). Further mouse studies with raspberry ellagitannins found them to be effective against TPA-induced ornithine decarboxylase activity, TPA-stimulated hydroperoxide production and TPA-stimulated DNA synthesis (47). Based on these findings, dietary administration of freeze-dried berries, in particular, black raspberries, is associated with inhibition of both the initiation and promotion/progression stages of carcinogenesis. The question still remains as to whether berries have any potential value at inhibiting tumors once they are fully developed.

In contrast to previous studies which examined chemopreventives for their anti-initiation and anti-promotion / progression activities, we decided to evaluate freeze-dried black raspberries for their therapeutic effects against N MBA-induced
carcinogenesis. Specifically, we determined if different doses of freeze-dried black raspberries had an effect at reducing the number and size of fully developed tumors in the rat esophagus that were induced with NMBA. Cellular proliferation rates, assessed by staining for proliferating cell nuclear antigen (PCNA), and animal survival statistics were also examined in this study. Effective agents that inhibit tumor development and delay metastasis and/or increase survival time could prove quite beneficial in individuals who already have cancer. This study is unique in that it is one of the few studies that has evaluated a chemopreventive agent for its potential therapeutic value.

4.3. Materials and Methods

4.3.1. Animals

Male F-344 rats, 5-6 weeks-of-age, were purchased from Harlan-Sprague Dawley (Indianapolis, IN). The animals were housed 3 per cage under standard conditions (20 ± 2°C; 50 ± 10% relative humidity; 12-hour light/dark cycles). Rats were maintained on modified AIN-76A diet containing 20% casein, 0.3% D,L-methionine, 52% cornstarch, 13% dextrose, 5% cellulose, 5% corn oil, 1% AIN vitamin mixture, 3.5% AIN salt mixture and 0.2% choline bitartrate (Dyets, Bethlehem, PA). Food and water were provided ad libitum throughout the bioassay. Hygienic conditions were maintained by twice weekly cage changes. Body weight and food consumption measurements were recorded bi-weekly after administration of berry diet and for the duration of the study.

4.3.2. Chemicals
N-Nitrosomethylbenzylamine (NMBA), obtained from Ash Stevens, Inc. (Detroit, MI), was determined to be greater than 98% pure by reverse phase HPLC analysis. Dimethyl sulfoxide (DMSO) was purchased from Sigma Chemical Company (St. Louis, MO).

4.3.3. Diet preparation

Black raspberries (*Rubus occidentalis*) of the Bristol variety were supplied by the Stokes Fruit Farm (Wilmington, OH). They were picked in July 2001, washed and frozen at -20°C within 24 hours of the time of picking. In May 2002, the frozen black raspberries were shipped to Van Drunen Farms (Momence, IL) where they were held frozen at -20°C until freeze-dried one week later. Freeze-drying was performed as described previously (48-50) with the exception that berry seeds were repulped and added back to the berry slurry before freeze-drying. Briefly, the black raspberries were washed and excess water drained. The berries were then placed at a depth of approximately 2-inches into metal trays and, using a Virtis freeze-drying unit, the berries were freeze-dried (lyophilized). The freeze-dried berries were then pulverized into a powder and the berry powder was shipped frozen to The Ohio State University (Columbus, OH) where it was stored at -20°C before use in the experiment. In addition, 100 gram samples of freeze-dried black raspberries were analyzed for compounds with known cancer inhibitory effects by Covance Laboratories (Madison, WI).

According to previous studies reported by Stoner and al., 5 and 10% berries were shown to possess an inhibitory effect against esophageal tumors (37, 50). Therefore, these were the doses chosen for this study. We chose an
additional dose of 20% berries based on the possibility that the larger dose of berries may offset tumor development to a greater degree than lower doses. On a bi-weekly basis, the freeze-dried black raspberries were mixed for 20 minutes in a modified AIN-76A diet at concentrations of 5, 10 and 20% using a Hobart mixer. The cornstarch in the diet was reduced by 5, 10, and 20% to provide a similar caloric intake in the berry diets and the control diet. On a bi-weekly basis, 5, 10, and 20% black raspberry diets and control diet were placed into glass feeding jars and fed to the animals.

4.3.4. Experimental design: Therapeutic bioassay

To evaluate the therapeutic effects of black raspberries on N MBA-induced tumorigenesis in the rat esophagus, animals were randomized into 6 groups and placed on AIN-76A diet (see Table 4.1). One week after acclimization to the facility four groups of rats were injected subcutaneously with N MBA (0.5 mg/kg body weight) once per week for fifteen weeks. Five rats from Group 3 were sacrificed at week 19 to determine if papillomas had developed. At 19 weeks, the animals had an average of 5 to 6 papillomas per esophagus (week 19). At that time, AIN-76A diets containing 5, 10, and 20% black raspberries were given until the animals were sacrificed at 26 weeks (see Figure 4.1). Group 1 was treated with 20% dimethyl sulfoxide (DMSO) in water, the solvent for N MBA. Group 2 was fed the high dose of black raspberries only, 20%. At week 26 all surviving rats were euthanized by CO₂ asphyxiation. The esophagus of each animal was opened longitudinally and the surface tumors were mapped, counted and measured. Lesions greater than 0.5 mm in diameter were considered to be
tumors. Assuming a spheroid shape, a volume estimate for each papilloma was calculated (51). One half of each esophagus was fixed on laminated cards in 10% neutral buffered formalin (NBF) to be processed for histopathology. The remaining one-half was stripped of the underlying layers of muscle and submucosa and immediately frozen in liquid nitrogen for subsequent molecular analysis. In addition, approximately 2 ml of blood was taken from each animal at sacrifice for analysis of berry components and antioxidant activity. In addition, sections of the liver, colon, kidney and spleen were harvested and fixed in NBF to evaluate possible toxicity of freeze-dried black raspberries.
<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. rats</th>
<th>Amount Admin. (ml)</th>
<th>NMBA Dose Admin. (mg/kg b.w.)</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMSO + H₂O</td>
<td>15</td>
<td>0.2</td>
<td>0</td>
<td>Control AIN-76A</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>15</td>
<td>0.2</td>
<td>0</td>
<td>AIN-76A + 10% BRB</td>
</tr>
<tr>
<td>3</td>
<td>NMBA</td>
<td>32</td>
<td>0.2</td>
<td>0.50</td>
<td>Control AIN-76A</td>
</tr>
<tr>
<td>4</td>
<td>NMBA</td>
<td>31</td>
<td>0.2</td>
<td>0.50</td>
<td>AIN-76A + 5% BRB</td>
</tr>
<tr>
<td>5</td>
<td>NMBA</td>
<td>31</td>
<td>0.2</td>
<td>0.50</td>
<td>AIN-76A + 10% BRB</td>
</tr>
<tr>
<td>6</td>
<td>NMBA</td>
<td>32</td>
<td>0.2</td>
<td>0.50</td>
<td>AIN-76A + 20% BRB</td>
</tr>
</tbody>
</table>

Abbreviations: NMBA, N-nitrosomethylbenzylamine; BW, body weight; BRB, freeze-dried black raspberries.

Table 4.1: Experimental design of therapeutic bioassay using freeze-dried black raspberries.
Figure 4.1: Experimental protocol for freeze-dried black raspberry therapeutic bioassay. Rats were acclimated to the facility for 2 weeks and then treated with NMBA at 0.50 mg/kg b.w. once per week for fifteen weeks. Two weeks later at 19 weeks, when they had an average of 5 to 6 papillomas per esophagus, NMBA-treated rats were started on diets containing either 5, 10, or 20% black raspberries.
4.3.5. Immunohistochemical analysis of cell proliferation and apoptosis

At the end of the study, the entire esophagus from five rats per group was stained for the cell proliferation marker, proliferating cell nuclear antigen (PCNA). The buffer used for antigen retrieval was 10 mM citrate (pH 6.0). The antigen retrieval time for the tissues was 10 minutes at 70% power. This was followed by blocking the tissues with 3% H$_2$O$_2$ for 20 minutes, casein for 15 minutes, goat serum for 20 minutes, and avidin/biotin for 30 minutes. The slides were then incubated with monoclonal mouse anti-PCNA antibody, the primary antibody, for 30 minutes (prediluted antibody from BioGenex, Inc). The secondary antibody, rat-absorbed link (biotinylated anti-immunoglobulin) and the label, streptavidin-horseradish peroxidase were each incubated for 20 minutes. To obtain biomarker visualization, the slides were incubated in 3, 3’-diaminobenzidine for 3.5 minutes. The slides were then counterstained with hematoxylin, dehydrated, and cover slipped with Permount (Fisher Scientific, Pittsburgh, PA). Colon from DMSO control groups and mouse antiserum was included in each run and served as the positive and negative control, respectively.

The same five esophagi used for PCNA were stained with caspase-3 as a measure of apoptosis. The buffer used for antigen retrieval was 10 mM citrate (pH 6.0). The antigen retrieval time for the tissues was 10 minutes at 70% power. This was followed by blocking these tissues with 3% H$_2$O$_2$ for 20 minutes, casein for 20 minutes, goat serum for 20 minutes, and avidin/biotin for 30 minutes. The slides were then incubated with monoclonal mouse caspase-3 antibody, the primary antibody, for 2 hours (diluted to 1:250 from Cell Signaling).
The secondary antibody, rat-absorbed link (biotinylated anti-immunoglobulin) and the label, streptavidin-horseradish peroxidase were each incubated for 30 minutes. To obtain biomarker visualization the slides were incubated in 3, 3'-diaminobenzidine for 6 minutes. The slides were then counterstained with hematoxylin, dehydrated, and cover slipped with Permount (Fisher Scientific, Pittsburgh, PA). Tonsilar and/or spleen tissue and mouse antiserum were included in each run and served as positive and negative controls, respectively.

4.3.6. Computer-assisted image analysis

Using a Nikon bright-field microscope mounted with a high-resolution spot camera, PCNA stained slides were viewed at x200. The camera was interfaced with a computer containing a matrox frame grabber board and image analysis software purchased by Simple PCI Imaging System by Complix, Inc. (Cranberry Township, PA). The basal layer of the esophagus was scanned every 10 fields (1,500-2,000 cells) and positively stained cells were quantified to determine the mean labeling index (LI). The LI is computed by dividing the area of positively stained nuclei by the total nuclear area. The LI is expressed as a percentage.

4.3.7. Statistical analysis

All statistical procedures were carried out using the NCSS 97 statistical software package (NCSS Statistical Software, Kaysville, UT). Body weight, food consumption, tumor multiplicity and tumor size data were analyzed for statistical significance ($P < 0.05$) using analysis of variance (ANOVA) followed by Neuman-Keuls’ multiple comparisons and Kruskal-Wallis tests. Differences in tumor
incidence were determined by Fisher's exact probability test and $\chi^2$ and Kruskal-Wallis tests.

For survival analysis, the means, medians and standard deviations of tumor numbers and average volumes were computed for each group defined by treatment (Vehicle, 20% BRB, NMBA, NMBA + 5% BRB, NMBA + 10% BRB, NMBA + 20% BRB). The groups were compared using a one-way analysis of variance (ANOVA). If the residuals were non-normal or had unequal variances, then a nonparametric Kruskal-Wallis test was performed. A significant group effect was followed up with multiple comparisons. Tukey's method of all pairwise comparisons was used. The overall alpha was set to 0.05. The mean survival time, standard error and median survival time were calculated for each group using the Kaplan-Meier method (52). If the overall test of equality of survival curves was significant, then follow-up multiple comparisons were performed using log rank tests.
4.4. Results

4.4.1 Therapeutic bioassay

There were no significant differences in mean body weight or food consumption (data not shown) for the animals prior to being fed berry diets. However, from weeks 23-26, there were significant reductions in body weight in rats treated with either NMBA only or NMBA and all three dietary concentrations of berries (Figure 4.2). These reductions in body weight may have been the result of increases in the size (especially in the pharyngeal region of the esophagus) and number of tumors throughout the esophagus thus reducing the passage of food through the esophagus. The therapeutic effects of freeze-dried black raspberries on NMBA-induced tumorigenesis in the rat esophagus are shown in Table 4.2. There were no significant differences in either tumor incidence, multiplicity or size in berry fed animals (Groups 4 and 5) versus animals treated with NMBA only (Group 3). Histopathological examination of sections of the liver, colon, stomach, kidney, and spleen from animals fed 20% berries indicated that the black raspberries did not elicit toxic effects in any of these organs.
Figure 4.2: Graph showing body weights of rats after being fed freeze-dried black raspberries at week 19. Group symbols are as follows: vehicle control (●); 20% freeze-dried black raspberries (■); NMBA control (▲); NMBA + 5% freeze-dried black raspberries (★); NMBA + 10% freeze-dried black raspberries (☆); NMBA + 20% freeze-dried black raspberries (●).
<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. rats</th>
<th>Tumor incidence (%)</th>
<th>Tumors/rat (mean ± SE)</th>
<th>Tumor size (mm³) (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>20% BRB</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>NMBA</td>
<td>16</td>
<td>100.0</td>
<td>8.6 ± 0.44</td>
<td>12.8 ± 1.6</td>
</tr>
<tr>
<td>4</td>
<td>NMBA + 5% BRB</td>
<td>20</td>
<td>100.0</td>
<td>6.2 ± 0.56</td>
<td>14.9 ± 8.2</td>
</tr>
<tr>
<td>5</td>
<td>NMBA + 10% BRB</td>
<td>21</td>
<td>100.0</td>
<td>9.3 ± 0.58</td>
<td>9.4 ± 1.8</td>
</tr>
<tr>
<td>6</td>
<td>NMBA + 20% BRB</td>
<td>22</td>
<td>100.0</td>
<td>8.9 ± 0.64</td>
<td>18.4 ± 5.3</td>
</tr>
</tbody>
</table>

Abbreviations: NMBA, N-nitrosomethylbenzylamine; BW, body weight; BRB, freeze-dried black raspberries.

Table 4.2: Effects of freeze-dried black raspberries on tumor development in NMBA treated F344 rats (Week 26 of the study).
4.4.2. Immunohistochemical staining

The PCNA LI’s for the experimental groups are depicted in Table 4.6, and photomicrographs are depicted in Figure 4.4. The average PCNA LI in the NMBA treated groups was 58.9 while the berry treated group average PCNA LI was 62.9, 70.1, and 73.3, respectively. In contrast, the PCNA LIs for untreated groups (Groups 1 and 2) ranged from 22.4 to 21.3% with no detectable differences between these two groups. As indicated in Table 4.6, dietary administration of freeze-dried black raspberries did not lower PCNA LIs. In fact, PCNA LIs increased as the doses increased. Since measuring apoptosis using caspase-3 staining needed additional optimization steps, this data was not included in the results and discussion portion of this paper.

4.4.3. Statistical analysis

Descriptive statistics for tumor number and volume are presented in Tables 4.3 and 4.4, respectively. Because Groups 1 (DMSO control) and Group 2 (20% black raspberries without NMBA) did not produce any tumors, they were excluded from the ANOVA. Assumptions of normality and equal variances of the residuals were considered when running ANOVA for tumor number. The results from the ANOVA indicate that there was a significant difference in tumor number between groups \([F=4.1, \text{df}=(3,105), p=0.009]\). Follow-up multiple comparisons suggest that there was only one significant difference in tumor number and that was between the NMBA + 5% BRB and NMBA + 10% BRB groups at the alpha=0.05 level.
Table 4.4 reports descriptive statistics for tumor volume. In this case, the residuals from the ANOVA were highly skewed and the variances were not equal. Therefore, a nonparametric Kruskal-Wallis test was performed. The results indicate that there were no significant differences between any of the groups with respect to tumor volume (p=0.101). The Kaplan-Meier survival estimates are presented in Table 4.5 and the survival curves are plotted in Figure 4.3. Overall, there were no significant differences in survival between the four groups (p=0.146).
<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of Rats</th>
<th>Mean</th>
<th>Median</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle control</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>20% BRB</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>NMBA alone</td>
<td>26</td>
<td>7.3</td>
<td>8</td>
<td>2.6</td>
</tr>
<tr>
<td>4</td>
<td>NMBA + 5% BRB,</td>
<td>29</td>
<td>6.1</td>
<td>6</td>
<td>2.4</td>
</tr>
<tr>
<td>5</td>
<td>NMBA + 10% BRB</td>
<td>24</td>
<td>8.8</td>
<td>9</td>
<td>2.9</td>
</tr>
<tr>
<td>6</td>
<td>NMBA + 20% BRB</td>
<td>30</td>
<td>7.9</td>
<td>8</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Table 4.3: Descriptive statistics for tumor number by treatment group for black raspberry therapeutic study.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of Rats</th>
<th>Mean</th>
<th>Median</th>
<th>SD</th>
<th>SE (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle control</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>20% BRB</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>NMBA alone</td>
<td>25</td>
<td>12.3</td>
<td>11.6</td>
<td>6.8</td>
<td>N/A</td>
</tr>
<tr>
<td>4</td>
<td>NMBA + 5% BRB,</td>
<td>29</td>
<td>14.1</td>
<td>5.9</td>
<td>31.1</td>
<td>N/A</td>
</tr>
<tr>
<td>5</td>
<td>NMBA + 10% BRB</td>
<td>22</td>
<td>9.5</td>
<td>9.2</td>
<td>7.4</td>
<td>N/A</td>
</tr>
<tr>
<td>6</td>
<td>NMBA + 20% BRB</td>
<td>29</td>
<td>16.4</td>
<td>8.9</td>
<td>20.9</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 4.4: Descriptive statistics for average tumor volume by treatment group for black raspberry therapeutic study.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of Rats</th>
<th>Mean</th>
<th>Median</th>
<th>SE (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle control</td>
<td>15</td>
<td>0</td>
<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>20% BRB</td>
<td>15</td>
<td>0</td>
<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>NMBA alone</td>
<td>29</td>
<td>42.5</td>
<td>N/A</td>
<td>1.9</td>
</tr>
<tr>
<td>4</td>
<td>NMBA + 5% BRB,</td>
<td>27</td>
<td>44.6</td>
<td>N/A</td>
<td>4.7</td>
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<tr>
<td>5</td>
<td>NMBA + 10% BRB</td>
<td>23</td>
<td>49.6</td>
<td>N/A</td>
<td>0.9</td>
</tr>
<tr>
<td>6</td>
<td>NMBA + 20% BRB</td>
<td>30</td>
<td>46.3</td>
<td>N/A</td>
<td>1.7</td>
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</table>

Table 4.5: Kaplan-Meier survival estimates by group for black raspberry therapeutic study.
<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>At start of study (Week 19)</th>
<th>At end of study (Week 26)</th>
<th>Calculated probability of survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle control</td>
<td>15</td>
<td>10</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>20% BRB</td>
<td>15</td>
<td>15</td>
<td>N/A</td>
</tr>
<tr>
<td>3</td>
<td>NMBA alone</td>
<td>32</td>
<td>16</td>
<td>50</td>
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<td>4</td>
<td>NMBA + 5% BRB</td>
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<td>20</td>
<td>64.5</td>
</tr>
<tr>
<td>5</td>
<td>NMBA + 10% BRB</td>
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<td>21</td>
<td>67.7</td>
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<tr>
<td>6</td>
<td>NMBA + 20% BRB</td>
<td>32</td>
<td>22</td>
<td>70.9</td>
</tr>
</tbody>
</table>

Abbreviations: NMBA, *N*-nitrosomethylbenzylamine; BW, body weight; BRB, freeze-dried black raspberries.

Table 4.6: Calculated probability of survival by group for black raspberry therapeutic study.

<table>
<thead>
<tr>
<th>Group</th>
<th>NMBA 0.50 mg/kg</th>
<th>Diet</th>
<th>% positive PCNA LI (± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>AIN-76A</td>
<td>22.4 (0.3)</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>20% BRB</td>
<td>21.3 (1.2)</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>NMBA</td>
<td>58.9 (3.1)</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>5% BRB</td>
<td>62.9 (2.1)</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>10% BRB</td>
<td>70.1 (2.0)</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>20% BRB</td>
<td>73.3 (2.1)</td>
</tr>
</tbody>
</table>

Abbreviations: NMBA, *N*-nitrosomethylbenzylamine; BW, body weight; BRB, freeze-dried black raspberries.

Table 4.7: Effects of freeze-dried black raspberries on PCNA LIs in the F344 rat esophagus (Week 26 of study).
Figure 4.3: Esophageal tissue sections stained with PCNA. Low level PCNA staining in normal esophageal epithelium (A); increased PCNA nuclear reactivity in dysplastic esophageal epithelium (B); intense PCNA staining in NMBA-induced papilloma tissue at week 26 (C); positive staining in rat colon control tissue (D).
4.5. Discussion

Studies in our laboratory have shown that the dietary administration of freeze-dried black raspberries prior to, during, and after NMBA treatment results in a significant inhibition of tumor development in the rat esophagus. The black raspberries were also effective at inhibiting NMBA-induced O\textsuperscript{6}-methylguanine adduct formation in esophageal DNA and in reducing the growth rate of premalignant cells in NMBA-treated animals. Based on the positive data obtained from studies using black raspberries in the rat esophagus model, a therapeutic study was conducted to determine whether or not freeze-dried black raspberries may be used for the treatment of NMBA-induced esophageal tumors in the rats. In this study, animals were fed 5, 10, and 20% of black raspberries in their diet after papilloma development and for the duration of the bioassay. The time course for the bioassay was limited to 26 weeks instead of 30 weeks due to a high number of animals dying in the NMBA-treated groups. The number and size of papillomas especially in Groups 5 and 6 prevented animals from consuming the diet thus leading to weight loss and eventually premature death. These observations support the results observed in the body weight graph (Figure 4.2). Animals fed 10% and 20% black raspberries, had no significant differences in tumor incidence, multiplicity or size when compared to NMBA-treated controls. Animals fed 5% black raspberries, however, had a significant reduction in tumor multiplicity when compared to NMBA-treated controls (Group 3).
The rationale for this study was to determine whether a chemopreventive agent might be useful when administered after tumors have already progressed. DFMO, for example, was effective when given to fully developed skin tumors in mice that were irradiated with UV (53). Most chemopreventive agents work by one of two ways. They can act either as blocking or suppressing agents (54-57). Blocking agents work by inhibiting the initiation of tumors by either influencing carcinogen metabolism, scavenging oxygen free radicals, or by enhancing DNA repair. Examples of blocking agents include berries, curcumin, ellagic acid, tea theaflavins, oltripraz, and diallyl sulfide. Suppressing agents work by inhibiting the promotion/progression stage of carcinogenesis. Their mechanisms of action include inhibition of cell growth, stimulation of apoptosis and/or cell differentiation, or inhibition of angiogenesis. Examples of suppressing agents include berries, calcium, sulindac, celecoxib, and ellagic acid (54, 55, 56).

The present study is the first to evaluate freeze-dried black raspberries, which exhibits both blocking and suppressing activity for potential beneficial therapeutic effects. Individuals who have esophageal SCC and a poor prognosis are most likely to benefit from a therapeutic agent that can increase survival time and reverse and/or inhibit the growth of malignant cells. Results from our study suggest that the dietary administration of freeze-dried black raspberries may have some therapeutic value, as evidenced by some reduction in tumor incidence and size and in survival of animals treated with the higher dose of black raspberries. However, this conclusion is tenuous all reductions were not significant, and PCNA cell proliferation rates were somewhat higher (although not
significantly so) in animals treated with berries plus NMBA verses those treated with berries only. In addition, the survival of animals was not improved in berry-fed groups. Interestingly, the concentrations (10 and 20%) of berries may be less effective than the lower (5%) concentration. Although the reasons for this are unknown, the following may occur: (1) Black raspberries may contain components that act as prooxidants at high enough concentrations; (2) Black raspberries may contain components that antagonize the inhibitory effects of other berry components leading, ultimately, to increased cell proliferation. The precise reasons for this observation have yet to be determined.

In this study, black raspberries may have been administered at such a late stage in tumor development that the events responsible for reducing cellular proliferation rates or other physiological effects; i.e. reducing angiogenesis, stimulating differentiation, could not be reversed. These studies should be expanded to include berries in conjunction with other chemopreventives; e.g., a COX-2 inhibitor or an inhibitor of ornithine decarboxylase to determine if the combined effects of these agents are more effective in tumor initiation and promoting animal survival.
4.6. References:


CHAPTER 5

DISCUSSION / SUMMARY

Cancer chemoprevention is defined as the use of natural agents or synthetic compounds that are able to block, reverse, or prevent the development of carcinogenesis which can eventually lead to invasive cancer (94, 96, 120). Chemopreventive agents can be classified into one of the following categories. They can either act as blocking agents or suppressing agents. Blocking agents play a role in the initiation stages of cancer by the following mechanisms: influencing carcinogen activation (by either blocking activation of carcinogens or increasing the detoxification of carcinogens); scavenging oxygen radicals; or enhancing DNA repair enzymes. Suppressing agents are effective in the promotion progression stages of carcinogenesis by a variety of mechanisms including: inhibiting polyamine metabolism, angiogenesis, basement membrane degradation, and oncogene activity. Suppressing agents can also induce terminal cell differentiation and apoptosis; modulate signal transduction and hormonal / growth factor activity; increase intracellular communication; restore immune responses, correct DNA methylation imbalances and decrease cellular proliferation rates. Both blocking agents and suppressing agents play a critical part in the cancer process. The process of cellular carcinogenesis is the
biological basis for the identification of chemopreventives, assessment of their activity, and ultimately the success or failure of a chemopreventive.

In a series of studies, our laboratory has evaluated a number of chemopreventive agents as inhibitors of against NMBA-induced esophageal tumorigenesis in the F344 rat model both in an anti-initiation and post-initiation scheme. These have included ‘food-based’ components such as tea theaflavins, curcumin and selected berries such as black raspberries and strawberries. In addition to ‘food-based’ chemopreventive agents, our laboratory has also examined single compounds such as oltripraz, isothiocyanates, diallyl sulfide, DFMO, 4-HPR, piroxicam and perillyl alcohol.

Work by Steinmetz et al. (103) have found that high levels of fruits and vegetable consumption, particularly fruits, are protective against cancer of the esophagus, oral cavity and larynx. Studies by Daniel et al. (65) have shown that various fruits are abundant in the plant polyphenol, ellagic acid which was shown to possess anti-mutagenic and anti-carcinogenic activity. Ellagic acid was shown to inhibit chemical carcinogenesis in selected organs by a variety of mechanisms. Furthermore, studies conducted by Stoner et al. (64) have found ellagic acid to be effective at inhibiting tumors in the rat esophagus. Yet, one cannot conclude that ellagic acid is responsible for the inhibitory effects of berries as shown by additional studies. Therefore, identifying specific components responsible for the protective effect observed in these studies is somewhat challenging. Nevertheless, using the food-based approach to chemoprevention is advantageous since foods contain multiple chemopreventive agents.
Black raspberries and strawberries contain a high amount of ellagic acid whereas blueberries contain a relatively small amount of this compound. Studies by Kresty et al. (73) have found that black raspberries and strawberries at a concentration of 5 and 10% in the diet were more effective than ellagic acid alone at inhibiting tumors in the rat esophagus in a 30-week anti-initiation bioassay. In addition, both berry types were effective in a post-initiation bioassay and at reducing the levels of O\textsuperscript{6}-methylguanine adducts. This was the case even when the amount of ellagic acid found in the berries was 10x less than an experiment using ellagic alone. Therefore, as described in the second chapter, we wanted to determine whether blueberries were effective chemopreventive at inhibiting tumors in the rat esophagus when they were administered in an anti-initiation bioassay. Not only are blueberries heavily consumed in many parts of the world but they also have been shown to contain high amounts of antioxidants, known chemopreventive agents and effective against improving memory and at inhibiting mammary carcinogenesis both in \textit{in vitro} and \textit{in vivo}. As detailed in Chapter 2, a series of bioassays including an anti-initiation and DNA adduct study were conducted to determine if blueberries had any chemopreventive activity when administered to rats at the same concentrations in which black raspberries and strawberries were shown to be effective. Dietary administration of 5 and 10% blueberries did not significantly reduce esophageal tumor incidence or multiplicity in this study. Tumor multiplicity increased somewhat in the 5 and 10%-treated groups when compared to N MBA-treated controls. The anti-initiation study was followed by an adduct study to determine whether blueberries would
reduce the levels of $0^6$-methylguanine adducts in NMBA-treated rats. Administration of 10% blueberries did not inhibit adduct formation following a single dose of NMBA at 0.50 mg/kg body weight. The results of this study are consistent with the observation that blueberries did not reduce tumor incidence, multiplicity or size in the rat esophagus.

As mentioned previously, nutrient analyses from all three berry types indicate that blueberries contain lower amounts of phenolics such as ferulic and p-coumeric acid and ellagic acid. It may be plausible that ellagic acid in combination with one or more phenolic compounds is responsible for the inhibitory activity of berries. In addition, blueberries contain a high amount of anthocyanins. These anthocyanins may be beneficial as well as harmful in some cases. This may have been the case for this study in which the high amount anthocyanins may exhibit a prooxidant effect similar to that seen in the beta-carotene study in human lung of tobacco smokers. Another explanation for blueberries inability to inhibit tumors may be the source of blueberries obtained. Studies by Kalt and al. show that lowbush blueberries were consistently higher in anthocyanins, total phenolics, and antioxidanat capacity, compared to highbush blueberries (117). We used high bush blueberries in our study.

As described in Chapter 3, a post-initiation study was conducted to determine whether DFMO, an irreversible inhibitor of ornithine decarboxylase (ODC), was effective at reducing tumors in NMBA-induced rat esophageal tumorigenesis. ODC is responsible for the conversion of ornithine to the polyamines; putrescine, spermidine, and spermine. A number of studies have
shown a correlation between elevated polyamine levels and tumor promotion / progression in animal and human tumors. Specifically, these polyamines are responsible for an increase in cellular proliferation rates that are observed in a number of neoplasias. Recently, our laboratory has been involved with identifying chemopreventive agents that inhibit tumor progression in the rat esophagus with the goal of using this as a model for squamous cell carcinoma development in former tobacco smokers. DFMO has shown to be one of the most promising suppressing agents in a number of tissues including the colon, bladder, breast, liver, stomach, skin and the esophagus (111). In addition, previous studies by Stoner et al. have shown this agent to be effective against esophageal tumors when given before, during and after carcinogen administration (121). Unlike other inhibitors, the chemical structure of DFMO is similar to ornithine. Therefore, DFMO competes with ornithine for the active site on the enzyme ODC. As a result of this inhibition, the level of polyamines are reduced thus decreasing cellular proliferation rates. At 25 weeks of this study, dietary administration of 1,000 ppm of DFMO nearly caused a significant reduction in tumor multiplicity. Although tumor incidence decreased somewhat, this decrease was not significant. The addition of animals to each treatment group may have resulted in a significant tumor inhibition. In contrast to the tumor data, DFMO did not significantly inhibit the development of preneoplastic lesions. Despite these findings, DFMO shows promise as a suppressing agent when administered in a post-initiation scheme. Current studies are underway to
determine if DFMO will act synergistically with black raspberries in inhibiting tumor development.

Although the mechanism is still being investigated, studies in our laboratory have demonstrated that black raspberries are effective chemopreventive agents when given in a pre and post-initiation bioassay in the NMBA-treated rat esophagus. However, we wanted to determine if black raspberries exhibited any therapeutic value to rats with fully developed esophageal papillomas. This hypothesis was tested in Chapter 4 where black raspberries were administered several weeks after NMBA-treatment. Although, the high dose of black raspberries (at a concentration of 20%) appeared to show an increase in survival of animals compared to NMBA-treatment alone at week 19, data obtained from week 26 showed that black raspberries did not possess any therapeutic value. Cellular proliferation data supported this conclusion. The dose of NMBA given during this study may have been too high. In addition, the timepoint in which the black raspberries were administered may have been too late to see an effect. We plan to do an additional study involving a lower dose of NMBA and an earlier timepoint of black raspberry administration to further evaluate their potential therapeutic effects.
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