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THE MECHANISM OF ACTION OF THE ANTICANCER EFFECTS OF SELENOMETHIONINE ON COLON CANCER

by

Antonio Thomas Baines

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Antonio Thomas Baines entitled The Mechanism of Action of the Anticancer Effects of Selenomethionine on Colon Cancer

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

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	Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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SIGNED: Antonio Thomas Baines

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"The race is not given to the swift or the strong, but to him that endureth until the end." This well known proverb describes my long journey to acquire an advanced degree in pharmacology and toxicology. It would be impossible to acknowledge every person that has contributed to my success from the beginning, but I will name a few that have had a major impact in helping me obtain a doctoral degree. First and foremost, I would like to give honor and praise God who deserves all the glory for this accomplishment. Earning this degree would have not been possible without His grace and mercy. Second, I want to thank my advisor, Dr. Mark Nelson, who allowed me the opportunity to grow from an eager, young, and timid graduate student to a serious and mature scientist. Thanks for not giving up on me. I would like to also thank my committee members: Drs. Margaret Briehl, Robert Dorr, David Earnest, and Claire Payne. They were excellent role models who shared interest and provided invaluable insight into my project. Special thanks go as well to Drs. Carlotta Groves and Dwayne Hill, who served as mentors early in my undergraduate years and continue to support me even to this day. The love and encouragement of all these mentors throughout the years have allowed me to achieve this degree. Thank You!

DEDICATION

First, I would like to dedicate the completion of this degree to my parents, Cleveland and Georgia Baines, who provided the love and support to accomplish this dream. Second, I want to honor my grandmom, Maggie Turner, whose prayers kept me going when I wanted to give up. Third, I want to dedicate this achievement to my high school biology teacher, Mr. George Waldenmaier, who nurtured my curiosity for the sciences. Finally, I want to dedicate this degree to Dr. Robert Baldwin, a plant pathologist, who showed me how teaching and research can go hand in hand.

Special Dedication goes to the late Dr. Larry Clark, an epidemiologist, who spent the majority of his research career trying to better understand how selenium might help the fight against cancer.

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ABSTRACT

Selenomethionine, an organic derivative of selenium, has been found to be the predominate selenium component of the dietary cancer chemopreventive agent selenized yeast that has been shown to decrease the incidence and mortality rate of lung, colon, and prostate cancers. Another organic selenium derivative found in the selenized yeast is Se-methylselenocysteine. However, the mechanism of action of the anti-cancer effects of these selenium compounds has yet to be identified. To evaluate the effects of these compounds on growth of the cancer cell types mentioned earlier, various cancer cell lines were treated with either compound. Both selenium compounds were able to induce growth inhibition and alterations in the cell cycle, with selenomethionine being the most potent.

Previously, our laboratory has shown that treating cancer cells with selenomethionine depleted polyamines. Polyamines are cations that are needed for various roles in growth and proliferation. To extend these findings to an *in vivo* model, we gave selenomethionine in the diet (1ppm and 2ppm) for 16 weeks to male F344 rats in the azoxymethane (AOM) rat colon carcinogenesis model. The results showed no significant changes in

colonic polyamine levels, however, there were significant changes in the development of microadenomas between control and treated groups. Selenomethionine was able to decrease the promotional effects of colon carcinogenesis through a polyamine-independent mechanism.

Next, we tested the hypothesis that selenomethionine might affect cell growth by mechanisms involving cyclooxygenases, specifically the inducible isoform COX-2. Cyclooxygenases (COX-1 and COX-2) are enzymes that metabolize arachidonic acid to various prostaglandins. The human adenocarcinoma cell lines HT-29 and HCA-7, that express variable levels of COX-2, were treated for up to 6 days with selenomethionine. Selenomethionine induced growth inhibition in both cell lines and decreased COX-2 protein expression in the treated groups. Also,prostaglandin(PG) E_2 levels were decreased in both treated groups at the latter timepoints. The HCA-7 cell line had a dose and time-dependent decrease in RNA levels treated with selenomethionine, whereas, no effects were observed on RNA expression of the HT-29 cell line. In summary, selenomethionine has chemopreventive effects in colon carcinogenesis by potentially modulating COX-2 proteins in cancer cells.

CHAPTER 1

INTRODUCTION

Colon Cancer

Colorectal cancer remains a significant health concern for much of the industrialized world being the second leading cause of cancer death among men and women in the United States (Ries et al., 1999). There were an estimated 93,800 new cases diagnosed and an estimated 47,700 deaths in 2000, accounting for about 11% of cancer deaths (Cancer Facts 2000).

Diagnosis often occurs at a late stage in the development of this malignancy, which reduces the likelihood of effective treatment. Symptoms of colorectal cancer include rectal bleeding, blood in the stool, and a change in bowel habits. Some risk factors include a personal or family history of colorectal cancer or polyps, physical inactivity, a high-fat and/or low-fiber diet, and an inadequate intake of fruits and vegetables (Cancer Facts 2000). It has been recommended by the American Cancer Society that beginning at age 50, men and women should begin colorectal screening with one of the following: a fecal occult blood test, a flexible sigmoidoscopy, or colonoscopy (Cancer Facts 2000). These tests provide the best opportunity

to detect the early stages of colorectal cancer which increases the possibility of successful treatment. The most common form of therapy for colorectal cancer is surgery, and it is frequently curative for cancers that have not metastasized. Other methods of treatment include chemotherapy and radiation. Chemotherapy, or chemotherapy plus radiation are given before or after surgery to patients whose cancer has advanced and invaded through the bowel wall or has spread to the lymph nodes. The 1 and 5 year relative survival rates for patients with colorectal cancer are 80% and 61%, respectively. This 5-year survival rate drops drastically, to 8% if distant metastases are involved. Due to the unsatisfactory outcome of these treatment methods, especially with advanced disease, much emphasis has been placed on developing better treatment and prevention measures.

Colorectal cancers can be classified as hereditary or as sporadic malignancies. Only 3-5 percent of all colorectal cancers have well-characterized inherited predispositions (Kinzler, K.W. and Volgestein, B., 1998). Two examples of the hereditary cancers include familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC). FAP is an autosomal dominant inherited disease in which affected individuals develop hundreds to thousands of adenomatous polyps

during the second and third decades of life. This inherited predisposition to colorectal cancer has been shown to be the result of the gene adenomatous polyposis coli (APC), located on chromosome 5q (Herrera et al., 1986). The inactivation of this tumor suppressor gene occurs not only in FAP, but in the early development of sporadic colorectal tumors as well. It has been estimated that at least 80% of colorectal tumors have a somatic mutation of the APC gene (Miyoshi et al., 1992). The second hereditary form of colon cancer, HNPCC or the Lynch syndrome, is also an autosomal dominant malignancy, but unlike FAP, these patients lack a marked increase in the number of precursor adenomas. It accounts for about 2-4 percent of colorectal cancer in the western world and starts to appear around the median age of 40 (Ponz de Leon et al., 1993). The majority of HNPCC patients inherit defects in DNA mismatch repair genes such as hMSH2 and hMLH1. This DNA mismatch repair-deficiency results in genetic instability and rapid progression to cancer (Lynch et al., 1996). Thus, in contrast to FAP, which mainly involves the initiation process of tumor development, the defect in HNPCC primarily affects tumor progression. In addition to colorectal cancer, HNPCC patients are at increased risk for cancers of the uterus, ovary, and brain (Lynch et al., 1996).

The vast majority of colorectal cancers do not have an easily recognized inherited component and therefore are considered sporadic. However, there is some suggestion of inherited susceptibility to relatives of patients with diagnosed sporadic colon cancers (Cannon-Albright et al., 1988). The accumulation of mutations and other abnormal genetic changes on various chromosomes have been shown to contribute to the development of colorectal cancer. These include mutations in tumor suppressor genes such as APC, DCC (deleted in colorectal cancer) gene, and p53, and oncogenes such as k-ras. These findings and others have made it possible to construct a model of the sequential genetic events in cancer initiation and progression (Volgestein, B. et al., 1993). Recently, studies have suggested that up-regulation of cyclooxygenase-2 (COX-2) which catalyzes the conversion of arachidonic acid to various prostaglandins, may be involved in tumor promotion of colon cancer (Oshima et al., 1996). A model of some of the genes involved in the development of colorectal cancer is shown in Figure 1.1. Along with the influence of genetics, environmental factors play a role in sporadic colon cancers as well. Epidemiologic studies indicate that diets high in animal fat and red meat, or low in fiber are associated with an increased risk for colorectal cancer. This could explain why Japanese

populations that move to the United States and adapt the Western-style diet, show a progressive increase in this malignancy (Haenszel et al., 1968).

Figure 1.1 A genetic model for colorectal tumorigenesis (modified from Fearon and Volgestein, 1990)

Normal Epithelium
↓ ←**APC, COX-2**
Dysplastic ACF
↓
Early Adenoma
↓ ←**k-RAS**
Intermediate Adenoma
↓ ←**DCC**
Late Adenoma
↓ ←**p53**
Carcinoma
↓
Metastasis

Selenium

The first recorded observation of selenium's (Se) biological effects was believed to be made by the world traveler Marco Polo concerning its toxicity to horses during the thirteenth century (1271-1295 AD) in Western China (Spallholz et al., 1994). This element was concentrated in the soil by the species of Astragalus plants, and when eaten by horses, resulted in loss of the animals' hoofs. This condition is believed to have been what is now

called "alkali disease". This disease was first associated with selenium in 1933 and is characterized by hair loss, loss of vitality, and deformation or loss of hoofs occurring in cattle, hogs, and horses consuming chronic and moderate levels of selenium found in plants and forage (Muth et al., 1967). Selenium is found in the soil and is absorbed best by plants as selenates and to a lesser degree as selenites. Plants metabolize selenium into various nonproteinaceous or proteinaceous amino acids, such as Se-methylselenocysteine and selenomethionine. Selenium is an essential dietary trace element due to various enzymatic reactions which utilize selenium as a cofactor. The United States National Research Council has calculated the Recommended Daily Allowance (RDA) for selenium to be 55μg/day for adult men and women. (Levander et al., 1991). Countries with selenium-rich soils, such as the United States, readily satisfy these requirements with their typical diets, but selenium-poor areas of the world (China, New Zealand, Scandinavia) do not have diets that furnish such required dietary selenium intakes. The National Research Council's Food and Nutrition Board recommends that 200μg Se per day is the maximum safe upper limit for adult human intake (Goyer et al, 1996). Symptoms of Se toxicity in humans include impaired vision, anorexia, confusion, paralysis,

and ultimately death from respiratory failure. The critical level for prevention of selenium deficiency is 20μg Se per day. Intake of less than 20μg/day may lead to the development of Keshan disease. This is an endemic cardiomyopathy that occurs most frequently in children under 15 years of age and in women of child-bearing age.

Selenium was recognized four decades ago as an essential nutrient based on its ability to serve interchangeably with vitamin E in the prevention of vascular or muscular problems in experimental animals (Schwarz et al., 1957). It was not until the discovery that the enzyme glutathione peroxidase contained Se as an essential component of its catalytic center (Rotrucket al., 1973), that the metabolic basis of its nutritional function become more clear. Glutathione peroxidase participates in the antioxidant protection of cells and this became the basis of the nutritional sparing of vitamin E by Se. This idea of the expanded nutritional function of selenium derived from various discoveries of several Se-dependent enzymes. These include glutathionine peroxidase isoforms (Chu et al., 1993) and other selenoenzymes and specific selenoproteins such as iodothyronine 5'-deiodinases (Croteau et al., 1995), thioredoxin reductase (Takashi and Stadtman, 1996), muscle selenoprotein W (Vendeland et al., 1995), and plasma selenoprotein P (Akesson et al.,

1994). Each of these selenoproteins contains Se in the form of selenocysteine, which is incorporated into the cysteine by co-translational modification. The current understanding is that the selenocysteine proteins are responsible for the nutritional functions of Se.

Metabolism of Selenium Compounds

The known pathways of selenium metabolism that are believed to be involved in anticancer activity are summarized in Figure 1.2 (Ipet al., 1998). Many selenium derivatives, organic and inorganic, are formed from this dynamic metabolic pathway. Animals have the capability to synthesize many different intermediary metabolites in the course of converting inorganic Se to organic forms and vice versa. Hydrogen selenide (H_2Se) is the common intermediate in both the assimilatory pathway for synthesis of selenoproteins, and for the synthesis of methylated selenium excretory products. It is formed from inorganic sodium selenite via the selenotrisulfide, selenodiglutathione (GS-Se-SG), through reduction by thiols and NADPH-dependent reductases (Ganther et al., 1971). Further metabolism leads to the formation of selenopersulfides (GSSeH) and then the final metabolite, hydrogen selenide. The following mechanism was

proposed by Ganther et al. (1968) for selenium reacting with glutathione leading to the formation of hydrogen selenite (Figure 1.3).

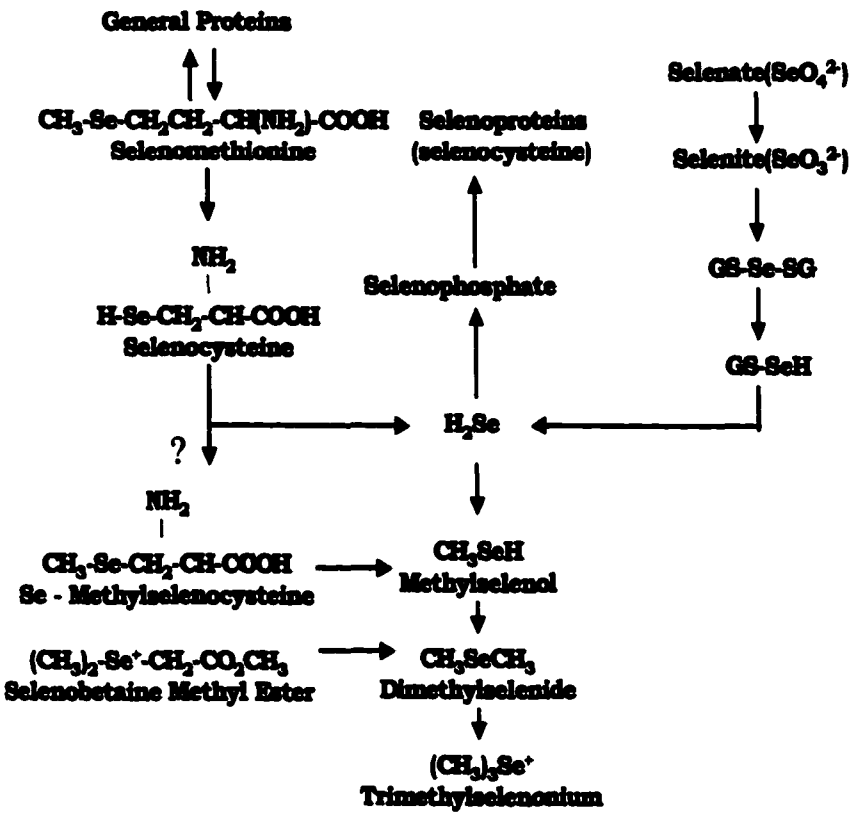


Figure 1.2 Pathways of Metabolism for Organic and Inorganic Selenium

$$4GSH + SeO_2 \rightarrow GSSeSG + GSSG + 2H_2O$$

$$4GSH + SeO_3 \rightarrow GSSeSG + 2OH + H_2O$$

Figure 1.3 Selenodiglutathione generation.

The toxicity of selenium compounds is believed to occur by production of reactive oxygen radicals resulting when selenite reacts with disulfide groups to form selenotrisulfides, similar to the reaction in figure 1.3. Selenotrisulfides can be reduced by excess thiols or by cellular glutathione reductase forming highly reactive selenopersulfides. Selenite formed in this way may then react with glutathione resulting in the formation of oxygen radicals which lead to cellular damage. The reaction of selenite with glutathione forming oxygen radicals is shown in Figure 1.4 (Seko et al., 1989).

$$\begin{array}{cccccccccc} & 4GSH & & GSSG & GSH & & GSSG & GSH & GSSG & O_2 \quad O_2^- \\ & & \curvearrowright & & & \curvearrowright & & & \curvearrowright & \quad\quad \curvearrowright \\ SeO_3 & \rightarrow & GSSeSG & \rightarrow & & GSSeH & \rightarrow & & H_2Se & \rightarrow \quad Se^0 \end{array}$$

Figure 1.4 Production of oxygen radicals from the reaction between selenite and glutathione.

Hydrogen selenide may also be released from the degradation of selenocysteine by β-lyase activity (Esaki et al., 1982). Selenomethionine

can be converted to hydrogen selenide via selenocystathione and selenocysteine by a trans-sulfuration reaction (Esaki et al., 1981). Selenocysteine can then form Se-methylselenocysteine by the addition of a methyl group and elemental selenium. This compound can be degraded into methylselenol, thus, entering the same metabolic pathway as the inorganic Se forms. After activation to selenophosphate, hydrogen selenide can provide Se for synthesis of selenoproteins (Veres et al., 1994). The known functions of Se as an essential element in animals are attributed to approximately 12 known mammalian selenoproteins (Gladyshev et al., 1998), all containing selenocysteine. Non-specific incorporation of Se into proteins occurs through substitution of selenomethionine for methionine.

Methylation is a major pathway for metabolism and enhancing excretion of Se in microbes, plants and animals, but demethylation to revert back to inorganic selenium can occur only in animals (Ganther et al., 1999). Sequential methylation of the inorganic hydrogen selenide by thiol methyltransferase (Hsieh et al., 1977) and the co-factor S-adenosylmethionine (SAM) results in the formation of methylselenol and dimethyl selenide. Further methylation by a thioether methyltransferase (Mozier et al., 1990) forms the trimethylselenonium ion. Excreted selenium

is in the form of dimethylselenide through expired air and trimethylselenonium in urine.

Anti-carcinogenic activity of selenium compounds

Three types of evidence have been used to support the belief that selenium has anticarcinogenic activity. One piece of evidence involves epidemiologic studies suggesting that a relatively low selenium status may be among the determinants of cancer risk. Shamberger and Frost (1969) proposed the hypothesis that Se status can be inversely related to the risk of some kinds of cancers. Shamberger and Willis (1971) showed that mortality due to lymphomas and cancers of the gastrointestinal tract, peritoneum, lung, and breast to be lower for men and women residing in areas of the United States that have moderate/high concentrations of selenium in forage crops in comparison to those residing in low-selenium forage areas. Another study in eastern Finland showed that mean serum Se concentration was significantly lower in cancer cases than in controls (Salonen et al., 1984). In a more recent study of Se status and cancer incidence, Clark et al. (1993 a,b) found that initial plasma Se concentrations of 1,738 Americans were inversely

related to subsequent risks of both non-melanoma skin cancer and colonic adenomatous polyps.

A second type of evidence that supports selenium being anticarcinogenic is from experimental carcinogenesis studies. Various animal models have been used to evaluate selenium's anticancer properties. Studies done by Ganther and Ip (1988, 1990, 1991) in the dimethylbenz(a)anthracene (DMBA)-induced mammary tumor model in the rat demonstrated that the selenium compounds selenite, selenocysteine, Se-methylselenocysteine, and dimethyl selenoxide could inhibit tumor formation. These investigators also utilized the rat methylnitrosourea (MNU) mammary tumor model demonstrating that the selenium compounds Se-methylselenocysteine, Se-propylselenocysteine, and Se-allylselenocysteine were active in reducing total tumor yield (Ip et al., 1999). Rao and Reddy (1996) used the rat azoxymethane (AOM) colon carcinogenesis model to show benzylselenocyanate, 1,4-phenylene bis (methylene) selenocyanate, sodium selenite, and dibenzyl diselenide could inhibit tumor growth. They also found a reduction in the formation of genetically predisposed lesions when they administered 1,4-phenylene bis (methylene) selenocyanate to the APC (min) mouse model (Rao et al., 2000). Studies conducted by Lin Yan et al.

(1999) demonstrated that selenomethionine supplementation reduced metastasis in mice injected with melanoma cells. Finally, Mukherjee et al (1996) showed that selenomethionine could reduce tumor incidence in 2-acetylaminofluorene-induced hepatocarcinogenesis in rats. These are just a few examples of the many studies of animal models that have been used to demonstrate selenium's anticarcinogenic properties.

A third piece of evidence that helps substantiate selenium's anticancer properties comes from various clinical trials. China has been the site for three clinical intervention trials that yielded results supportive of anticancer efficacy of selenium. The first two were reported in a 1995 review article (An et al., 1995). The first study conducted in Qidong, Shandong Province, China involved the use of a daily supplement of200μg Se as Se-enriched yeast. Findings from this trial indicated that Se treatment eliminated liver cancer incidence among hepatitis surface-antigen carriers and reduced over a 2-year period, the annual incidences of that cancer among first degree relatives. The second study employed table salt fortified with 15 ppm Se as sodium selenite in the same provincial area. The results indicated that liver cancer incidence dropped in villages provided the Se-fortified salt compared to the controls. The third study was conducted in an area with a high

prevalence of esophageal cancer, in Linxian, Henan Province, China. Those results (Li et al., 1993) suggested that a treatment containing Se (50μg Se/day as Se-enriched yeast plus vitamin E and B-carotene), had modest protective effects against total and stomach cancer mortality among subjects in the general population. A more recent, decade-long, clinical trial was conducted in the United States that suggested anticancer efficacy for Se (Clark et al., 1996). It consisted of a double-blind, placebo-controlled trial of 1,312 older Americans with histories of basal and/or squamous cell carcinomas of the skin and that lived in the low forage selenium area of the U.S. The results showed that the use of a daily oral supplement of Se-enriched yeast (200ug Se/day) did not affect the primary endpoint, the risk of recurrent skin cancers. However, in comparison to controls, Se treatment was associated with lower incidences of other tumors including total non-skin cancers, cancers of the lung, colon-rectum, and prostate, as well as overall cancer mortality rate.

Selenomethionine

The organic selenium derivative, selenomethionine, was identified in plant proteins in the 1950-60's, and shown to be produced by various strains

of bacteria and marine algae grown in Se-containing media (Shrift et al., 1973). Cereals and forage crops convert Se mainly into selenomethionine and incorporate it into proteins in place of methionine (Met). Figure 1.5 shows the biosynthesis of selenomethionine by brewers yeast, plants, and marine algae. It is not required for growth of plants but is produced along with methionine in quantities depending on the amount of bioavailable Se. The replacement of methionine by selenomethionine can influence enzymatic activity, especially if selenomethionine replaces methionine in the vicinity of its active site. For example, β-galactosidase of *E. coli* has been shown to have over half of the 150 methionine residues replaced by selenomethionine, resulting in inactivity of this enzyme (Huber et al., 1967).

Although animals and humans are unable to synthesize selenomethionine, this compound can be readily incorporated into body proteins after being taken into the body from the diet. This allows selenium to be stored in the organism and reversibly released by normal metabolic processes. Ingested selenomethionine is absorbed in the small intestine via the Na+ - dependent neutral amino acid transport system (Vendeland et al., 1994). Selenomethionine is activated initially by adenosylation, demethylated, and converted to selenocysteine (Kajander, E.O., 1991). Also, metabolism of

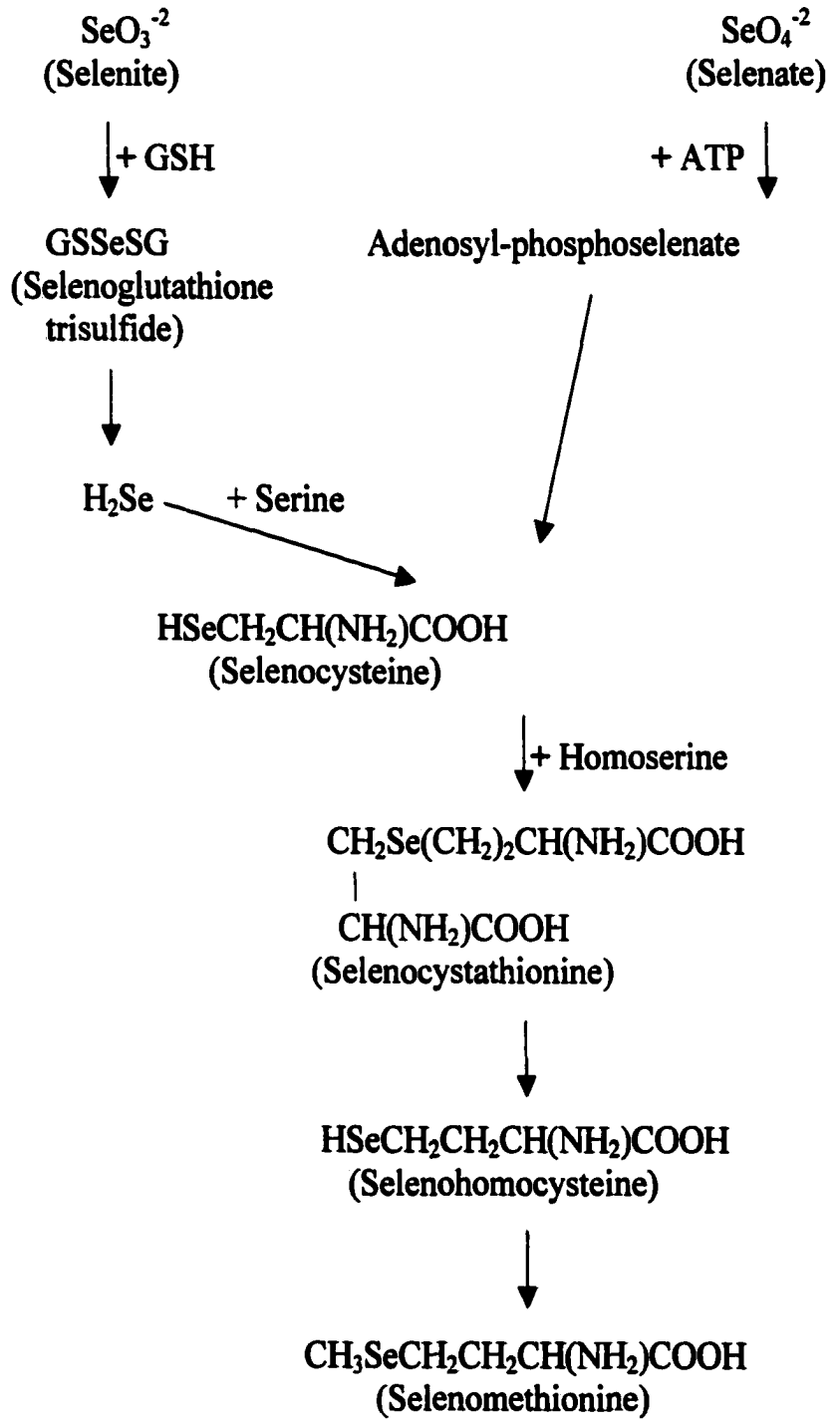


Figure 1.5 Selenomethionine biosynthesis in plants, marine algae, and brewers yeast.

selenomethionine to methylselenol (CH_3SeH) occurs by γ-lyase action (Nakamuro, K., 1997). The rate of degradation for this processes compound has been shown to be rapid (Patterson et al., 1989) and is dependent on vitamin B_6 status. This is due to the involvement of B_6-dependent enzymes in selenomethionine metabolism (Soda et al., 1999). Incorporation into organs with high rates of protein synthesis, such as the skeletal muscles, developing erythrocytes, liver, gastrointestinal mucosa, etc., occurs to the remainder of selenomethionine that is not immediately metabolized. Thus, selenomethionine is utilized and reutilized extensively (Swanson et al., 1991).

Selenized yeast (Selenoprecise@, Cypress Systems) was administered to participants in the Nutritional Prevention of Cancer Trial, discussed earlier. Analysis of the highly selenized yeast revealed that about 70% of the selenium was in the form of seleno-L-methionine. Other forms of organic selenium derivatives found in trace amounts in the yeast included selenocystine, Se-methylselenocysteine, and selenoethionine. However, the anticarcinogenic mechanism of action of many of these selenium compounds, including selenomethionine, has yet to be elucidated. Ip et al. (1999) reported that selenium compounds that are able to generate a steady

stream of methylated metabolites, particularly monomethylated selenium metabolites, are likely to have good anticarcinogenic potential. Also in 1991, Yan and Spallholz's studies found that selenate and selenomethionine do not generate superoxide (Yan et al., 1993). This was supported by Beutler et al. (1975) where they found that selenate and selenomethionine possessed no glutathione peroxidase catalytic activity, unlike selenite and selenocysteine. Overall, these results indicate that another mechanism may exist for the anticancer effects of selenomethionine other than the traditional role of selenium being involved with the antioxidant glutathione peroxidase.

Polyamines

There exists a connection between selenium metabolism and polyamine biosynthesis in that they both require the cofactor S-adenosylmethionine (SAM). Polyamines are a ubiquitous group of low molecular weight polycationic molecules that play a central role in cellular growth and differentiation (Pegg et al., 1988). The primary polyamines found in mammalian cells and tissues include putrescine, spermidine, and spermine. Depletion of polyamines in mammalian cells results in an

inhibition of growth of neoplastic cells both *in vitro* and *in vivo* (Pegg et al., 1988). When quiescent cells are stimulated, the level of ornithine decarboxylase (ODC), the rate limiting enzyme in polyamine biosynthesis, rises, resulting in an increase in polyamine levels prior to DNA, RNA, and/or protein synthesis (Tabor et al., 1976, Janne et al., 1978, Williams-Ashman et al., 1980).

Polyamines are involved in many steps of DNA, RNA, and protein synthesis. Tumor cells exhibit a high requirement for these molecules in order to sustain cellular growth. This is achieved by *de novo* synthesis and enhanced captation from the extracellular environment (Pegg et al., 1988). Polyamines have been shown to regulate the cell's preparation for DNA synthesis, affect the rate of formation of the DNA replication fork, and stimulate or inhibit various enzymes involved in DNA synthesis or metabolism (Tabor et al., 1984). Also, polyamines are thought to affect chain termination of protein biosynthesis (Tabor et al., 1984).

Polyamines are cations derived from the amino acids arginine and methionine (Pegg et al., 1986). Figure 1.6 shows the steps involved in polyamine biosynthesis starting with ornithine. Putrescine is formed from the conversion of ornithine by ornithine decarboxylase. Spermidine and

spermine are then produced by the enzymes spermidine synthase and spermine synthase, using S-adenosylmethionine (SAM) as a co-factor for both of these reactions. Once SAM has been decarboxylated to donate its aminopropyl group to form spermidine and spermine, it is committed to polyamine production and is no longer available for other methyl transfer reactions (Pegg et al., 1988), (Pegg et al., 1987). Putrescine is degraded by diamine oxidase or excreted from the cell when its levels are higher than the amount required for polyamine production (Pegg et al., 1988). The rate limiting factor for polyamine production is the supply of decarboxylated SAM (Pegg et al., 1988). After the decarboxylated SAM has donated an aminopropyl group to form spermidine and spermine, 5'methylthioadenosine (MTA) is produced. MTA is then usually rapidly degraded to adenine monophosphate (AMP) and methionine by the enzyme MTA phosphorylase (Williams-Ashman et al., 1982), (Schlenk et al.,1983). Spermidine and spermine can be converted back into spermine and putrescine, respectively, by the enzymes spermine/spermidine N-acetyltransferase (SSAT) and polyamine oxidase. It is noteworthy that there is no alternative pathway for polyamine biosynthesis in mammalian cells (Porter et al., 1987).

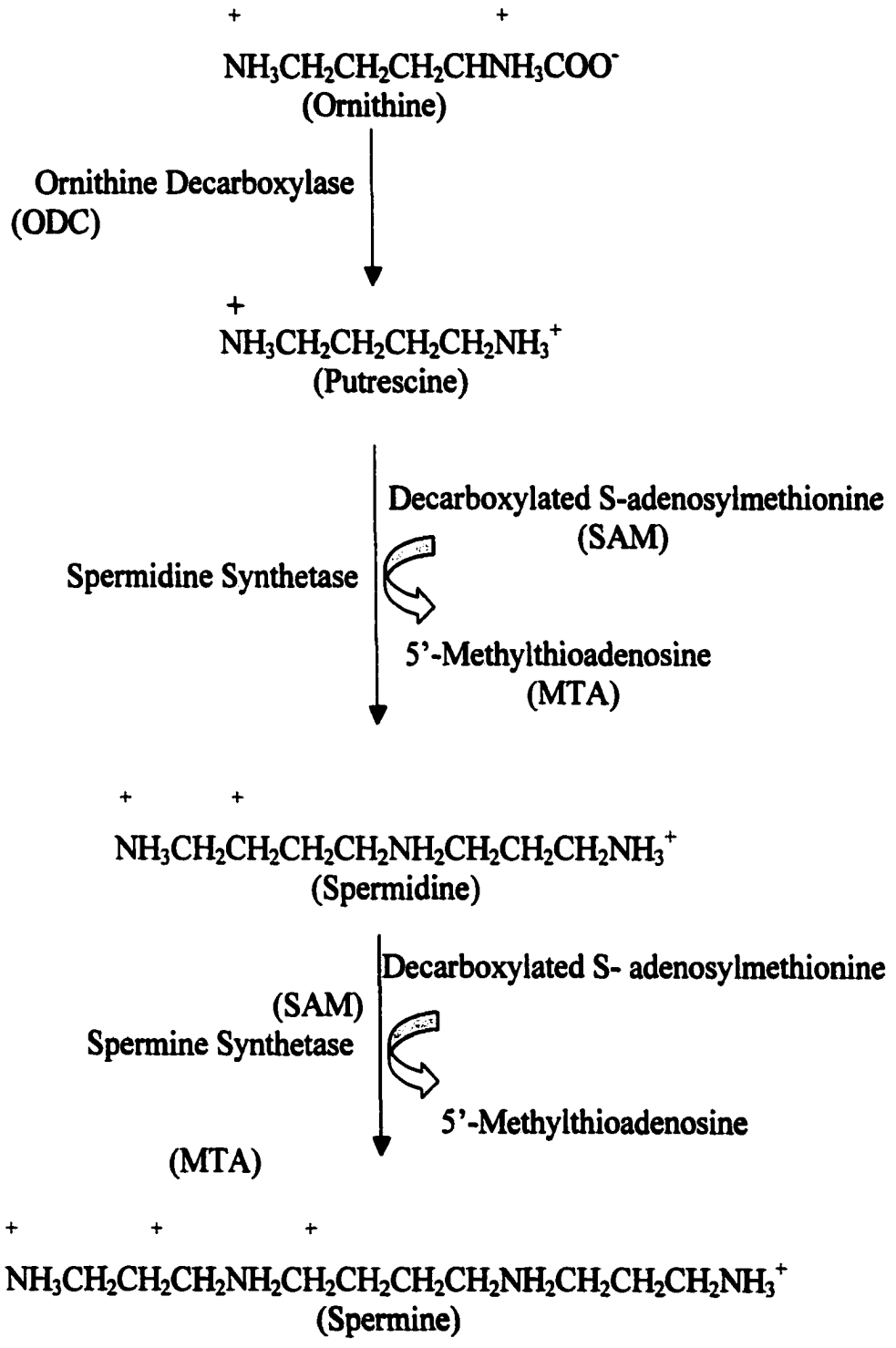


Figure 1.6 Polyamine Biosynthesis.

Selenium Metabolism and Polyamine Biosynthesis

The common link between selenium metabolism and polyamine biosynthesis is that they both require SAM as a cofactor (Figure 1.7). It was therefore hypothesized that if SAM was being utilized for selenium methylation reactions, it would be unavailable for polyamine production. If this was the case, then polyamine levels would decrease and the cancer cells would stop proliferating after selenium administration. As cancerous cells have a faster proliferation rate than normal cells, their polyamine levels are higher, and therefore cancer cells should be more susceptible to any level of inhibition of polyamine production.

Kajander et al. (1980) showed that the cytotoxicity induced by selenomethionine in several established malignant cell lines was dependent on the methionine content of the media in which the cell lines were cultured. An increase of cytotoxicity was observed commensurately with a decrease in methionine content of the media. Kajander et al.(1980) concluded that there was a direct or indirect involvement of SAM in selenomethionine cytotoxicity. McGarrity et al. (1993) demonstrated that sodium selenite administration to the human colon cancer cell line HT-29 decreased ODC activity, putrescine content, and cell growth. It was further noted that at the

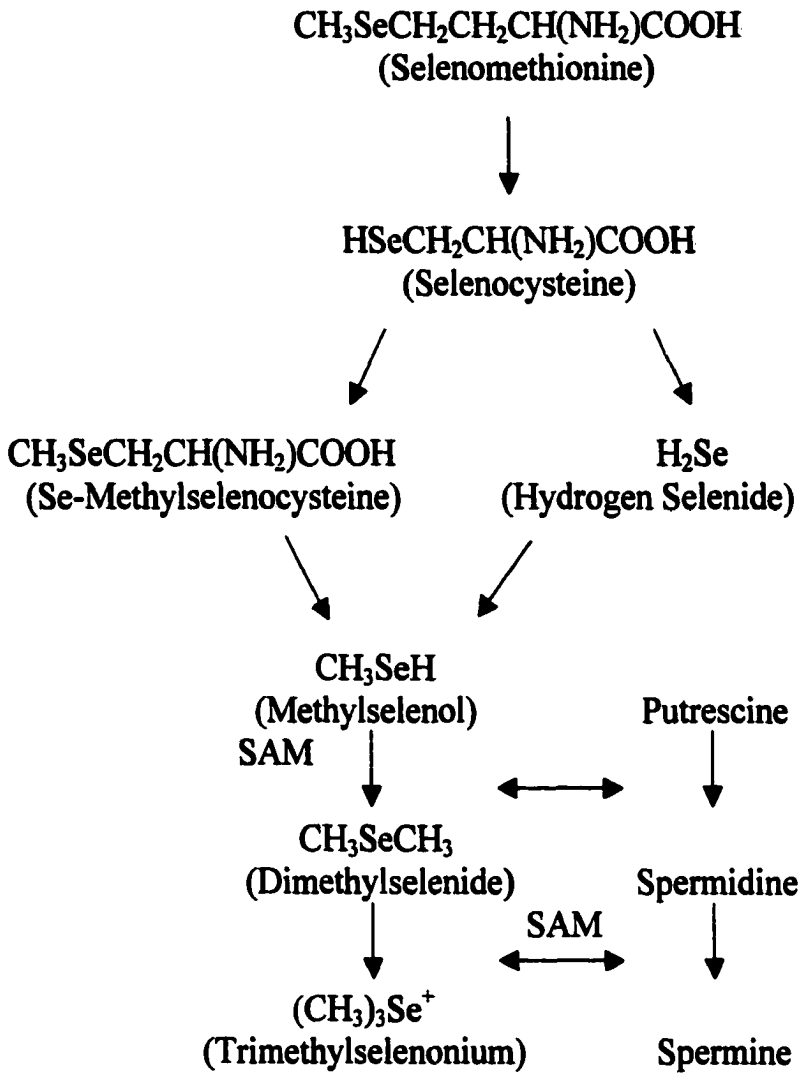


Figure 1.7 Sites of SAM Participation in Polyamine Biosynthesis and Selenomethionine Metabolism.

concentration of selenite used, a significant increase in SAM levels was observed. The increase of SAM was correlated with its lack of participation as a co-factor in the production of spermidine and spermine. However, it is

important to emphasize that the mechanism of action for selenite, involving glutathione and formation of hydroxyl radicals, differs from the mechanism of action of selenomethionine.

Redman et al. (1997) showed that selenomethionine-induced apoptosis and mitotic alterations in human A-549 lung and HT-29 colon cancer cell lines correlated with a depletion in polyamines. At 24 and 72 hr, polyamine content of both tumor cell lines was decreased at doses of selenomethionine that inhibited 50% of normal growth. When exogenous spermine was added back to the cells, apoptosis was prevented. They concluded that at least part of the anti-carcinogenic effects of selenium supplementation might be due to a depletion of polyamine levels, leading to an induction in apoptosis and perturbations in the cell cycle.

Cyclooxygenases

Selenium and non-steroidal antiinflammatory drugs (NSAIDs) are being utilized alone and in combination in cancer chemoprevention trials against colon cancer. One possible mechanism for these agents may involve affecting enzymes needed to produce prostaglandins. The key regulatory enzymes in the eicosanoid biosynthetic pathway that is responsible for

producing various proinflammatory prostaglandins are cyclooxygenases (COX). Also known as prostaglandin H synthase, it catalyzes the formation of the prostanglandin endoperoxide from arachidonic acid to PGG_2 and PGH_2. PGH_2 can then be converted to a variety of pharmacologically active eicosanoids that include PGE_2, PGD_2, PGE_2, PGI_2, and thromboxane $(TX)A_2$ as shown in Figure 1.8. The downstream enzymatic machinery present in a particular cell type determines the array of prostaglandins being produced. Prostaglandins are present in a wide variety of human tissues (Mead et al., 1986), and play a central role in the inflammatory response and regulation of other critical physiological responses. For example, prostaglandins are involved in blood clotting, ovulation, initiation of labor, bone metabolism, nerve growth and development, wound healing, kidney function, blood vessel tone, and immune responses (Mead et al., 1986). Two classes of prostaglandin receptors exist to transduce signals upon binding of ligand, the G-coupled cytoplasmic receptor class (i.e. EP1-4 for PGE_2) and the nuclear peroxisome proliferator-activated receptor (PPAR) class (i.e. $PPAR_\alpha$, $PPAR_\gamma$, $PPAR_\delta$), which acts directly as transcription factors upon ligand binding (Forman et al., 1997).

Earlier studies done on cell growth signaling pathways and inflammatory factors led to the identification of two isoforms of cyclooxygenases, COX-1 and COX-2 (Raz et al., 1988). It was revealed that while both enzymes carry out essentially the same catalytic reaction and have similar primary protein structures (Smith et al, 1996), there are some differences. The

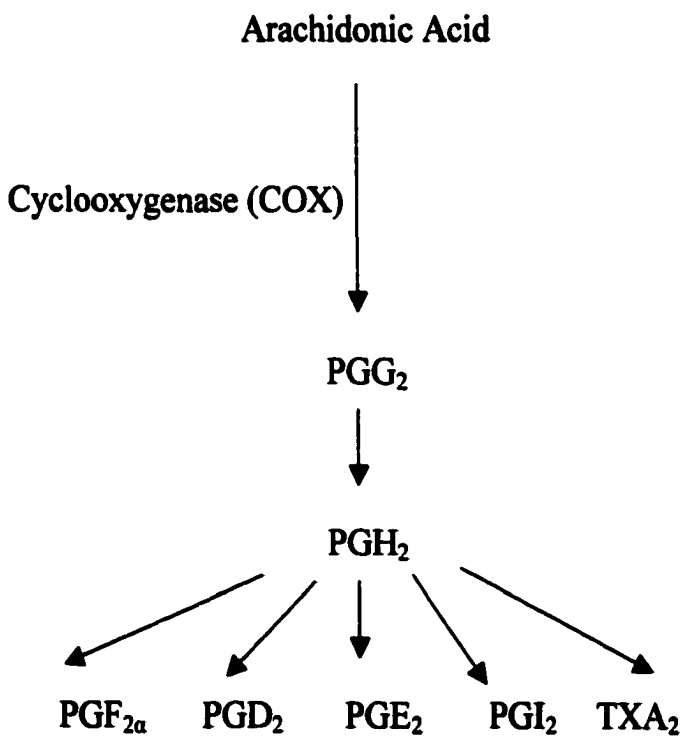


Figure 1.8 Schematic diagram for the conversion of arachidonic acid to prostaglandins and other eicosanoids by the cyclooxygenase enzymes.

isoform COX-1 is constitutively expressed and mediates many of the 'housekeeping' effects of COX, while COX-2 is inducible and mediates many of the inflammatory effects of COX. The functional roles of each isoform are consistent with tissue expression patterns. Nearly all normal tissues have been found to express COX-1 with low to undetectable levels of COX-2. Another difference between isoforms is the regions regulating gene expression. The promoter and enhancer regions regulating COX-2 contain a variety of response elements (i.e. NF-κB) that have been shown to explain some of its inducibility by hormones, growth factors, phorbol esters, cAMP, inflammatory factors, and cytokines. Less is known about the elements involved in COX-1 gene regulation. The COX-1 gene lacks a TATA box whereas the COX-2 gene contains a TATA box (Kraemer, S.A., 1992) (Appleby, S.B., 1994). COX-1 is generally induced by cell quiescence and differentiation whereas COX-2 is an immediate-early response gene. Also, there are major differences in mRNA splicing, stability, and translational efficiency between COX-1 and COX-2 (DuBois et al., 1998). For example, the regulation of COX-2 at the mRNA level appears to be an important mechanism by which some physiological mediators, such as corticosteroids, act to regulate prostanglandin synthesis during immunosuppression. In

summary, the COX-1 and COX-2 genes are regulated by two independent and different systems even though the enzymatic reaction they catalyze is identical.

Cyclooxygenase-2 and Colon Cancer

A widely-used class of drugs that is an inhibitor of COX enzymes is the non-steroidal antiinflammatory drugs (NSAIDs), with aspirin being the prototype member of this class. Two case-controlled drug surveillance studies and one large cohort study found that patients reporting regular aspirin use had a reduced incidence or decreased death rate from colorectal cancer (Rosenberg et al., 1991, Kune et al., 1988, Thun et al., 1991). Several different NSAIDs have been shown to reduce the formation of both colon adenomatous polyps (precursor lesion of colon cancer) and cancers in experimental animals given known carcinogens (Tanakaet al., 1991, Pollard et al., 1989, Reddy et al., 1992). These and other studies demonstrate the potential for NSAIDs as chemopreventive agents for colorectal cancer. However, prolonged use of NSAIDs can lead to gastrointestinal (GI) side effects that are probably due to inhibition of COX-1 regulated gastric prostaglandin production which is needed for GI mucosal maintenance

(Sheng et al., 1997). In determining the molecular basis for the chemopreventive effects of NSAIDs, it was discovered that both human and animal colorectal tumors expressed high levels of COX-2. In contrast, the normal intestinal mucosa had low to undetectable levels of COX-2 expression (Kutchera et al., 1996, Sano et al., 1995). Approximately 70-80% of human colorectal carcinomas have increased levels of COX-2 comparable to normal colon tissue (Eberhart, et al., 1994, Chapole et al., 2000). These findings led to the hypothesis that COX-2 may play a role in colon cancer growth and progression. Evidence from various genetic and pharmacological studies has demonstrated that COX-2 plays a causal role in the development of colorectal cancer (Williams et al., 1999, Gupta et al., 1998). In addition, selective inhibitors of COX-2 also retard the growth of polyps, highlighting the potential clinical utility of these drugs for the prevention and/or treatment of colorectal cancer (Steinbach et al., 2000). However, the mechanism of how COX-2 directly contributes to colon cancer is not fully understood.

STATEMENT OF THE PROBLEM

One of the areas of cancer research that has brought about new possibilities and innovative approaches to preventing colorectal cancer is chemoprevention. In chemoprevention, noncytotoxic nutrients or pharmacological compounds that protect against the development and progression of mutant clones of malignant cells are used to either inhibit or reverse carcinogenesis (Greenwald et al., 1995). One of these nutrients that has been of interest to our laboratory group is the dietary trace element selenium. We have focused on the organic derivatives of selenium due to their lesser toxic effects in comparison to the inorganic forms and their presence in the high selenized yeast used in a successful human intervention trial. Chemical analysis confirmed that the predominant form of selenium in the dietary supplementation was selenomethionine, with a lesser percentage being Se-methylselenocysteine and other selenopeptides. We decided to use both of these compounds as tools to better understand the mechanism of action of how the organic derivatives of selenium may be working to retard or prevent the development and progression of colorectal cancer. This

research which focused on selenium and colon cancer was conducted using three major hypotheses.

The first hypothesis for this dissertation is that selenomethionine and Se-methylselenocysteine can inhibit growth in various tumor cell lines.

The second hypothesis is that selenomethionine's anticancer effects are due to depletion of polyamine levels that results in decreased colonic tumor growth *in vivo*.

The third hypothesis is that selenomethionine may exert its antiproliferative effects by decreasing prostaglandin E_2 production through inhibition of cyclooxygenases, specifically cyclooxygenase 2 (COX-2).

To test the first hypothesis with both organoselenium compounds, two specific aims were designed. The first specific aim was to determine the concentrations that inhibited 50% growth (IC_{50}) for the D, L and L-enantiomer of selenomethionine and Se-methylselenocysteine. Three representative human cancer cell lines of the prostate (DU-145), lung (A-549), and colon (HT-29) were used. These cancers were chosen due to the findings of decreased incidence in these specific cancer types in the

nutritional interventional trial with selenium supplementation. To determine the IC_{50}'s for both selenium compounds in these cell lines, the Sulforhodamine B cytotoxicity assay was utilized.

The second specific aim was to compare the *in vitro* effects, in regards to change in cell number and alterations in the cell cycle, of selenomethionine and Se-methylselenocysteine on the three different cell lines. Cells were grown in a 6-day *in vitro* experiment and analyzed at 2 day intervals. Viable cells were counted by trypan blue exclusion and analyzed by flow cytometry for alterations in the cell cycle.

For the second hypothesis, previous studies from our group had already shown decreased polyamine levels in selenomethionine-treated colon cancer cells. To test this hypothesis *in vivo*, we extend these findings to the azoxymethane (AOM)-induced rat colon carcinogenesis model. The rats were initiated with the carcinogen (AOM) and fed special-made diets that contained 1 or 2 ppm of selenomethionine for 16 weeks. At the end of the study, the colon was excised and examined for aberrant crypt foci (precursor lesions), tumor counts, and polyamine measurements.

For the third hypothesis concerning cyclooxygenases, three specific aims were designed. The first specific aim was to analyze the protein expression

of cyclooxygenases in selenomethionine-treated colon cancer cells using Western blot analysis. The two cell lines used were HT-29 and HCA-7 cells, with the latter constitutively expressing large amounts of COX-2 compared to HT-29 cells. The second specific aim was to measure prostaglandin E_2 (PGE_2) levels in the media of selenomethionine-treated colon cancer cells. This is one of the major prostaglandins secreted by colon cancer cells. The prostaglandin levels were measured using an enzyme immunoassay (EIA) kit. The third specific aim was to analyze the RNA expression of COX-2 in selenomethionine-treated colon cancer cells using Northern Blot analysis.

CHAPTER 2

INHIBITION OF GROWTH OF SEVERAL HUMAN TUMOR CELL LINES BY SELENOMETHIONINE AND SE-METHYLSELENOCYSTEINE

INTRODUCTION

Selenium supplementation has become important due to its anticarcinogenic properties discovered through epidemiology, animal models, and *in vitro* studies. Various epidemiology studies have suggested an inverse relationship between environmental selenium levels and cancer incidence. Clark et al. (1991) reported that cancer mortality rates were significantly lower in intermediate-selenium and high-selenium counties in comparison to low-selenium counties for total cancer and cancers of the lung, colon, rectum, bladder, etc. From a ten year intervention trial on dietary selenium supplementation, Clark et al. (1996) showed a decrease in total cancer incidence in patients with a history of non-melanoma skin cancer. Also, there was a significant reduction in the incidence of lung, colon, and prostate cancers with the dietary selenized yeast supplement. The active selenium metabolite as well as the mechanism of action involved in the cancer chemopreventive effects of dietary selenium supplementation are still a subject for investigation.

Selenium is known to function as a component of several selenoenzymes, especially the glutathione peroxidase located in the cytosol or in cellular membranes (Flohe et al., 1973). These peroxidases protect the cell from oxidative damage. Studies with various forms of selenium have demonstrated that mechanisms other than interactions with glutathione peroxidases are involved in the observed anticarcinogenic properties. In 1991, Yan and Spallholz reported that unlike selenite and selenocysteine, selenomethionine does not react with glutathione and does not generate superoxide (Yan et al., 1991). Beutler et al. (1975) also found that selenomethionine possess no glutathionine peroxidase activity, unlike selenite and selenocysteine. Another organic form of selenium, Se-methylselenocysteine, was found by Lu et al. (1995) to be able to induce a pattern of cellular and molecular responses that is distinct from that caused by selenite. These data suggest that different pathways affecting cell proliferation and cell death are modulated depending on whether selenium metabolism is initiated by hydrogen selenide (contributed by selenite) or methylselenol (contributed by Se-methylselenocysteine). Methylation is an important pathway of selenium metabolism and the generation of mono-methylated forms of selenium appears to be a prominent feature of these

organic compounds possessing anti-carcinogenic activity (Ganther and Lawrence, 1997). Overall, these results indicate that selenomethionine and Se-methylselenocysteine have a completely different mechanism of cytotoxicity than other selenium compounds such as selenite.

Both of these organic derivatives of selenium, selenomethionine and Se-methylselenocysteine, were found to be in the selenized yeast that was used in the human intervention trial. Chemical analysis of the dietary supplementation determined that approximately 80-90% of the selenium content was in the form of selenomethionine and 0.5% was in the form of Se-methylselenocysteine (Schrauzer et al., 1998). To gain further insight into the observations seen in the dietary selenium supplementation study by Clark et al. (1996), we compared the relative efficacy of selenomethionine and Se-methylselenocysteine on growth inhibition in three human tumor cell lines established from organ sites (colon, lung, and prostate) shown to be affected in the human intervention trial. The first objective was to find the optimum growth of each cell line. The second objective was to determine the concentration of both selenium compounds that resulted in 50% growth inhibition (IC_{50}) for each cell line. The third objective was to use the IC_{50} to

drug the cell lines in a 6-day *in vitro* experiment to evaluate effects on cell number and cell cycle.

MATERIALS AND METHODS

Cell Culture

The human colon cancer cell line HT-29, the lung carcinoma cell line A-549, and the prostate carcinoma DU-145 were obtained from American Type Culture Collection, ATCC (Rockville, Maryland). The lines were maintained in monolayer cultures in RPMI 1640 media (Mediatech, Inc., Herndon, VA) with 5% fetal bovine serum (Hyclone Bioproducts, Loggan, UT), 2 mM L-glutamine (Gibco BRL, Grand Island, NY), 50 I.U./mL penicillin (Gibco BRL) and 50 μg/mL streptomycin (Gibco BRL). The passages for HT-29 ranged from 130-140, from 87-97 for A-549, and from 67-77 for DU-145. All cell lines were grown in a humidified incubator with 95% air and 5% CO_2 at 37°C. Cell lines were monitored for the absence of mycoplasmas using the Mycoplasma Detection Kit (Boehringer Mannheim, Indianapolis, IN) by the Tissue Culture Core of the Arizona Cancer Center. Subculturing was done at subconfluent densities with a phosphate buffered saline solution of 0.25% trypsin (Gibco BRL).

Sulfurhodamine B Assay

Cell growth was determined using the sulfurhodamine B (SRB) protein colorimetric stain assay (Skehan et al., 1990). Optimum cell number per well was determined for each cell line over a seven day period. For the lung cancer cell line, A-549, 250 cells/well were plated; for the DU-145 cell line, 1200 cells/well were plated; and the HT-29 cell line, 500 cells/well were plated. Ninety-six well microtiter plates were used for plating the cell lines and the cells were subsequently incubated for 24 hours prior to the addition of compounds to be tested. All selenium compounds used in these experiments were soluble in the media used for the cell line. Each compound was tested over a concentration range of six 10-fold dilutions. The media-only controls and each of the compounds were tested in a minimum of six wells per experiment. After an additional six days of culture, the viable cells were fixed to the bottom of each well with cold 50% trichloroacetic acid (TCA) (Sigma, St. Louis, MO) at a final concentration of 10%. The plates were kept at 4°C for 1 hour and the supernatant was then aspirated and the plates were washed with deionized water three times. The SRB (Sigma, St. Louis, MO) solution was prepared to 0.4% (wt/vol) in 1% acetic acid (Mallinckrodt, Paris, KY). The SRB (50 μL) was added to each

well and the protein of the cells was stained for 10 minutes at room temperature. Unbound SRB was removed by washing with 1% acetic acid followed by air drying. Bound stain was solubilized with 50 mM unbuffered Tris (Intermountain Scientific, Bountiful, UT) and optical density (OD) was measured by an automated spectrophotometer (Biomek 1000@, Beckman, Fullerton, CA) at a single wavelength of 540 nm.

Determination of IC_{50} Values

For each compound, a minimum of three experiments measuring the growth inhibition of all the cell lines was completed. Percent cell survival was determined by taking the optical density of each treated cell row and dividing it by the optical density of the media (vehicle) treated cells in the same 96 well plate. The inhibitory effect of the compound at each concentration was expressed as a percentage (mean OD of treated cells/mean OD of control or media treated cells) x 100. Each concentration of compound was then plotted on the X- axis vs. the percent inhibitoryeffect on the Y- axis using Excel. In this manner, a dose-response curve was generated and the IC_{50} was estimated by interpolation from the inhibitory effects measured at each concentration tested.

Cell Treatments

The methodology and treatment with selenium is depicted in Figure 2.0. All cell lines were maintained in T-175 flasks as previously described. Cells were in log phase growth when they were subcultured into 100 mm tissue culture plates for treatments. Each cell line was plated at the following densities: A-549 and DU-145 = 4 x 10^4 cells/plate, HT-29 = 8 x 10^4 cells/plate. After being passed into the plates, the cells were allowed to adhere for 24 hours before treatments. Compounds and concentrations used to treat the cell lines were as follows:

A-549 cell line: Seleno-L-methionine (Sigma, St. Louis) IC_{50} = 23 uM;

L-Se-methylselenocysteine (Sigma, St. Louis) IC_{50} = 28 uM.

DU-145 cell line: Seleno-L-methionine IC_{50}=53uM;

L-Se-methylselenocysteine IC_{50} = 22 uM.

HT-29 cell line: Seleno-L-methionine IC_{50} = 32 uM;

L-Se-methylselenocysteine IC_{50} = 25 uM.

The concentrations above were obtained from the results of the IC_{50} determinations of the compounds (Results Section). Selenomethionine and Se-methylselenocysteine were solubilized in media. Cells were collected at their respective time points for analysis every 2 days after treatment up to 6

days. For the cells that were not collected until later during the 6-day model, the old media was aspirated and the cells were provided with fresh media and treatment. There was a total volume of 10 mL in each plate. For harvesting of plates, media was aspirated and cells were enzymatically removed from the plate with a phosphate buffered saline solution of 0.25% Trypsin. To determine effects on growth, the number of viable cells was determined based on Trypan Blue (Sigma) exclusion. Trypan Blue was mixed in an equal volume with resuspended cells and viable (dye-excluding) cells were counted on a hemacytometer. Three different experiments were conducted to obtain average cell numbers for each time point for the cell lines for the control and treated groups. The average cell numbers were plotted against time (days) using Excel software.

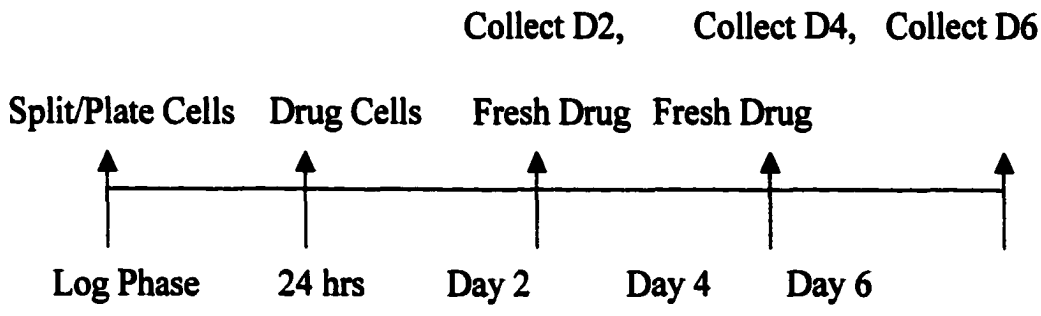


FIGURE 2.0 Design of the 6-day in-vitro repeated dosing xperiments used for administering selenium compounds to tumor cells.

Cell Cycle Analysis

Flow cytometric analysis of the cell cycle was performed according to the protocol described by Krishan et al. (1975). After harvesting the cells as described earlier, cells were counted using Trypan Blue exclusion and 0.5-1.0 x 10^6 cells were pelleted in a 15 mL conical centrifuge tube. Pellets were resuspended in 1mL of Krishan's buffer (0.1% sodium citrate, 0.02 mg/mL RNase A, 0.3% NP-40, and 50 ug/mL propidium iodide) at a pH of 7.4. Following resuspension in Krishan's buffer, samples were vortexed and stored at 4°C, wrapped in foil until analysis. Cell samples were sent to the Flow Cytometry Service of the Arizona Cancer Center for analysis of the cell cycle phase and results were expressed as percentages.

Statistical Methods

Several methods were used to fit the curve to each of the experiments, but an exponential model was the best fit selected most of the time (Orgin 4.0 for Windows 95, Northampton, MA). After the coefficients of the fitting function were obtained, the concentration causing a 50% reduction in the wells was estimated. The resulting IC_{50} from each experiment was then compared in an analysis of variance (ANOVA) for differences among the selenium compounds for every type of cell. The statistical significance

between all pairs of compounds was assessed using Tukey's multiple comparisons test (SAS 6.12 for Windows 95, Cary, NC).

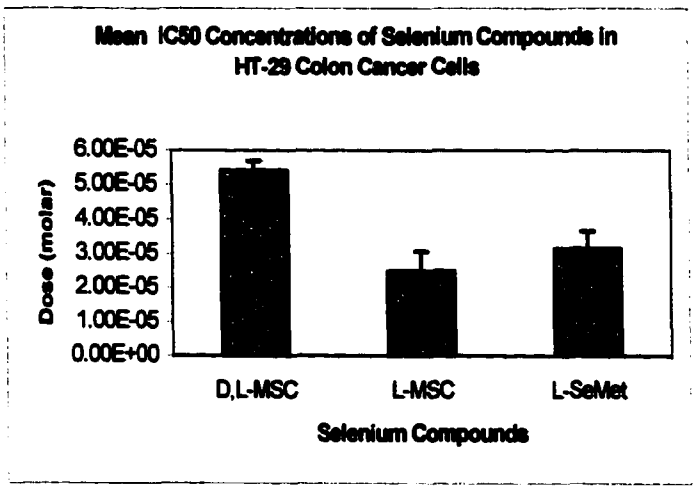
RESULTS

The Determination of IC_{50} Values of Selenomethionine and Se-Methylselenocysteine on Tumor Cells

The concentrations of seleno-L-methionine (SeMet) and L and D,L-Se-methylselenocysteine (MSC) that inhibited 50% tumor cell growth (IC_{50}) were investigated by the SRB assay. These three organic derivatives of selenium were used against the following three human tumor cell lines: A-549 lung carcinoma, HT-29 colon carcinoma, and DU-145 prostate carcinoma. A total of three experiments were performed with each selenium compound for each cell line. Figure 2.1 shows the mean IC_{50}s for the three selenium derivatives against the human colon, lung, and prostate cancer cell lines. The lower the IC_{50} value, the more potent the selenium compound is as an inhibitor of tumor cell growth. The L-isoforms of both selenium compounds were found to be more potent than D,L-Se-methylselenocysteine in the colon and lung cancer cells. There was no significant difference between the two L-isoforms of the two selenium compounds in both of these tumor cell lines. In the prostate cancer cell line, L-Se-methylselenocysteine

was found to be slightly more potent than the other two selenium compounds tested. There was no significant difference in potency between seleno-L-methionine and D,L-Se-methylselenocysteine for this cell line.

A.



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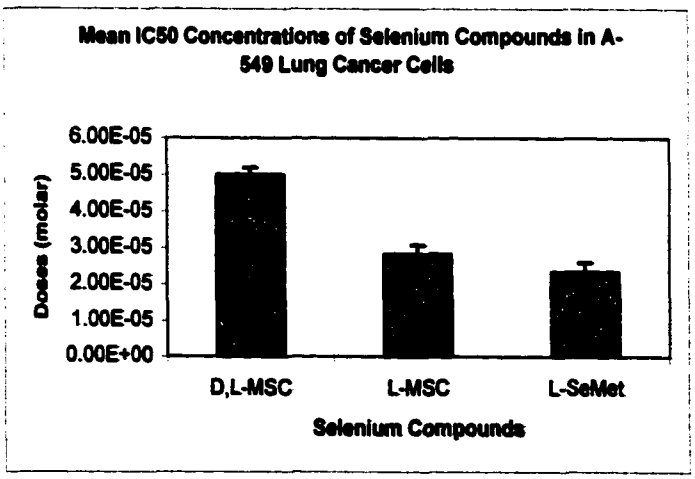

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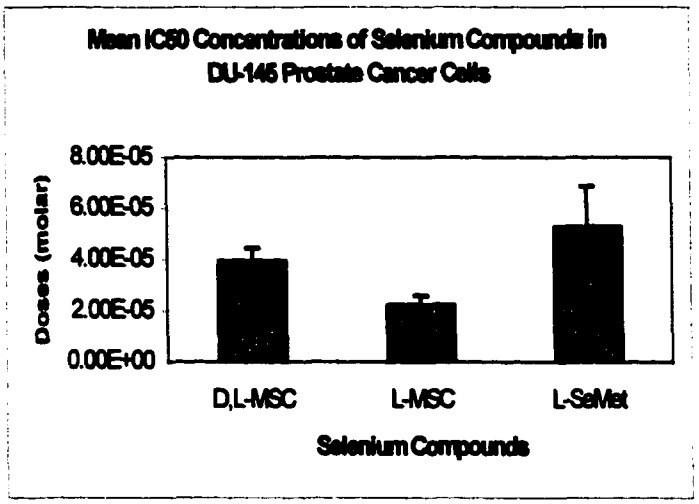
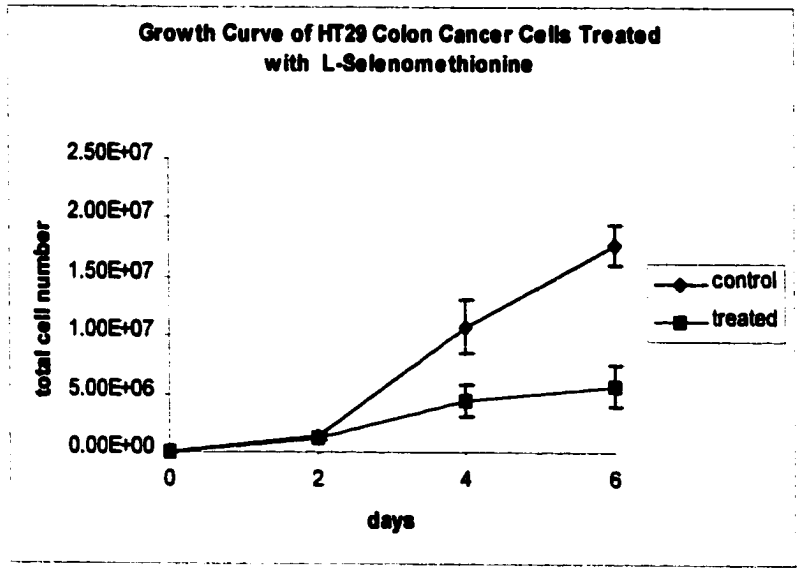


Figure 2.1 Mean IC_{50}s for the three selenium derivatives in A) HT-29 Colon; B) A-549 Lung; and C) DU-145 Prostate Cancer Cells, Mean ± S.D. (n=3 experiments for each cell line for each compound; cells were treated for 6 days)

The Effects of Selenomethionine and Se-Methylselenocysteine on Tumor Cell Growth

The ability of seleno-L-methionine and L-Se-methylselenocysteine to inhibit tumor cell growth was investigated with a 6-day repeated dose *in vitro* experiment. The L-isoform of these compounds were used in this study due to being more biologically active than the D,L-isoforms. Figures 2.2 shows the effects of both compounds on the growth of HT-29 colon cancer cells. For selenomethionine, there is a significant inhibition of growth in the treated cells, beginning at day 4. For Se-methylselenocysteine, the significant inhibition seems to occur later, around day 6. Figure 2.3 evaluates the effects of both selenium compounds on the growth of A-549 lung cancer cells. Selenomethionine and Se-methylselenocysteine were both found to significantly inhibit growth as early as day 4. Figure 2.4 demonstrates the effects of both selenium compounds on the growth of DU-145 prostate cancer cells. Only selenomethionine was found to significantly inhibit the growth of this cell line, as early as day 4. There was no difference in growth between the control and Se-methylselenocysteine-treated cells in the 6-day model.

A.



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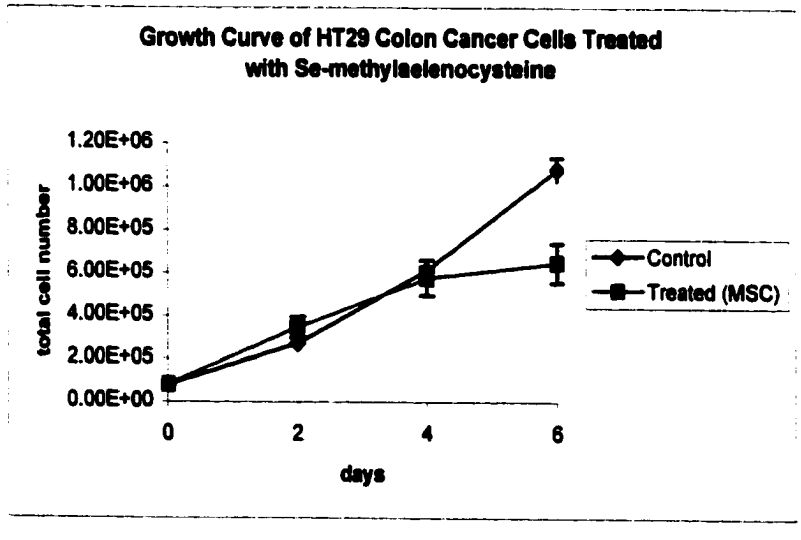


Figure 2.2 Growth curves of HT-29 colon cancer cells treated with the IC_{50} of **A)** SeMet or **B)** MSC up to 6 days. Mean ± S.E. (n = 3 experiments)

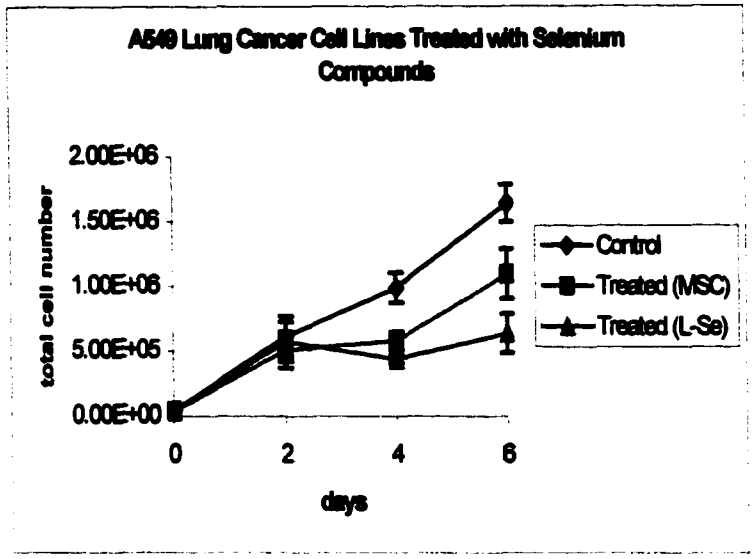


Figure 2.3 Growth curves of A-549 lung cancer cells treated with the IC_{50} of SeMet or MSC. Mean ± S.E. (n = 3 experiments for each compound)

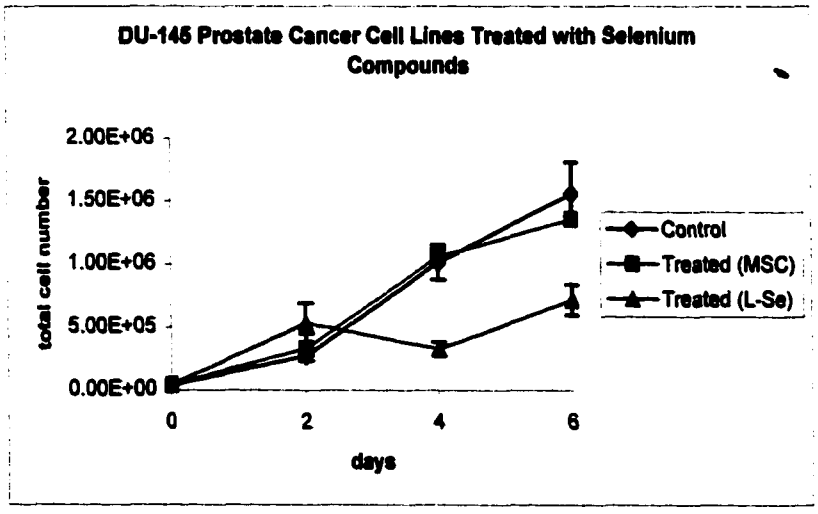


Figure 2.4 Growth curves of DU-145 prostate cancer cells treated with the IC_{50} of SeMet and MSC. Mean ± S.E. (n = 3 experiments for each compound)

The Effects of Selenomethionine and Se-Methylselenocysteine on the Cell Cycle of Tumor Cells

The ability of selenomethionine and Se-methylselenocysteine to cause changes in the cell cycle of asynchronous cancer cells was evaluated in the 6-day *in vitro* model. Only the day 6 timepoint for each cell line was used for the analysis since that corresponded to the most growth inhibition. Cell cycle changes were demonstrated in the HT-29 colon cancer cells (Table 2.1) and the A-549 lung cancer cells (Table 2.3). In the HT-29 cells treated with selenomethionine cells, there was a 3-fold increase of cells in the G2/M phase of the cell cycle and a decrease in the other phases compared to the control. In the A-549 selenomethionine-treated cells, there was a slight increase in the number of cells in the S phase of the cell cycle compared to control (from 19% to 28%). In contrast, Se-methylselenocysteine administration caused an increase in G0/G1 and a 2-fold decrease in the G2/M phase of the cell cycle (Table 2.3). There were no changes observed with the DU-145 prostate cancer cells treated with the selenium compounds (Table 2.4).

Table 2.1 Average percentage of HT-29 colon cancer cells in the different phases of the cell cycle at day 6.

	G0/G1	S	G2/M
C	71.1	23.1	5.8
SeMet	66.5	15.5	18.1

n = 3 experiments

Table 2.2 Average percentage of HT-29 colon cancer cells in the different phases of the cell cycle at day 6.

	G0/G1	S	G2/M
C	68.4	23.3	8.3
MSC	68.9	21.0	10.0

n = 3 experiments

Table 2.3 Average percentage of A549 lung cancer cells in the different phases of the cell cycle at day 6.

	G0/G1	S	G2/M
C	69.5	18.6	11.9
MSC	81.0	13.8	5.2
SeMet	63.0	27.9	9.1

n = 3 experiments

Table 2.4 Average percentage of DU-145 prostate cancer cells in the different phases of the cell cycle at day 6.

	G0/G1	S	G2/M
C	63.9	25.7	10.4
MSC	62.2	27.6	10.2
SeMet	58.5	28.9	12.6

n = 3 experiments

DISCUSSION

From the ten year intervention trial on dietary selenium conducted by Clark et al. (1996), there were significant reductions in the incidence of prostate, colon, and lung cancers in the selenium-treated group. Chemical analysis of the selenium-supplemented yeast tablet used in the study showed that between 70-80% of the selenium was in the form of selenomethionine, with the remainder being in the form of other selenopeptides (Schrauzer et al., 1998). One of those selenopeptides was found to be Se-methylselenocysteine, a naturally-occurring, monomethylated form of selenium that does not get incorporated into proteins, as selenomethionine does. These findings have led to the quest of understanding the mechanisms involved in selenium's effects on the results of the dietary selenium clinical trial. The purpose of this present study was to evaluate the growth inhibitory effects of two organoselenium compounds, selenomethionine and Se-methylselenocysteine, on lung, colon, and prostate cancer cell lines.

One of the major objectives of this present study was discovering the IC_{50} for each compound with each cell line and finding if those concentrations could inhibit growth in the cancer cell lines in a time-course *in vitro* model. The IC_{50} for the D,L- isoform of selenomethionine was found

from a previous study (Redman et al., 1997). Redman also demonstrated that cancer cells were more sensitive to selenomethionine than normal fibroblasts (Redman et al., 1997). From the inhibitory concentrations in this present study, there was no significant difference between the L-enantiomers of selenomethionine and Se-methylselenocysteine in the lung and colon cancer cell lines in the SRB assay. In the repeated dose experiments, both compounds were able to inhibit growth, although selenomethionine was found to be more potent. This is in contrast with the results by Ip et al. (2000) showing Se-methylselenocysteine being more superior than selenomethionine. One major difference between the studies that could explain the differences in outcome is the cell type used. Ip's work evaluated selenium's anticancer effects against mammary cancer and used rat mammary cell lines. Each cell type may metabolize selenium compounds differently depending on the levels of various enzymes, such as β-lyase. This enzyme is responsible for metabolizing Se-methyselenocysteine into methyselenol. In the present study, L-Se-methylselenocysteine was found to have a more potent IC_{50} than the other compounds in the prostate cancer cell line, but there was no inhibition observed in the 6-day*in vitro* repeated dose model. Only selenomethionine was able to significantly inhibit growth in

comparison to the control in this cell line. The observation of the IC_{50} of L-Se-methylselenocysteine not causing growth inhibition was unexpected and further experiments are warranted to clarify this finding.

In reference to the cell cycle, the growth inhibition observed in the selenomethionine-treated HT-29 colon cancer cells could potentially be explained by the accumulation of cells in the G2/M checkpoint. Also, cell cycle changes could explain the inhibitory effects observed by both selenium compounds in the A-549 lung cancer cells. Medina et al. (1997) demonstrated that inhibition of mammary tumor cell growth by Se-methylselenocysteine is mediated by alterations in progression of cells in the cell cycle, resulting in a cell cycle arrest. This accumulation of cells in a particular phase of the cell cycle can retard growth or cause cells to undergo apoptosis. Redman et al (1997) demonstrated that D,L-selenomethionine caused growth inhibition and apoptosis in HT-29 and A-549 cancer cells at earlier timepoints. Because some of the selenium compounds have no cell cycle effects, (e.g. DU-145s), mechanisms other than cell cycle arrest must be responsible for the growth inhibition. One possible mechanism might involve regulating genes involved in growth and apoptosis, such as growth arrest and DNA-damage (gadd) genes. Kaeck et al. (1997) showed that

selenium compounds could increase the expression of various gadd genes such as gadd45 in growth arrested cells.

In summary, the IC_{50} for selenomethionine and Se-methylselenocysteine were found to cause growth inhibition and some cell cycle changes in a 6-day *in vitro* repeated dosing model using three different human cancer cell lines. These data suggests that in human cancer cell lines, representing tumor types affected by selenium supplementation, selenomethionine and Se-methylselenocysteine have different mechanisms of action. This supports the finding in the Clark et al. (1996) study that selenium supplementation can have anticancer effects on various types of cancers.

CHAPTER 3

THE EFFECTS OF DIETARY SELENOMETHIONINE ON POLYAMINES AND AZOXYMETHANE-INDUCED ABERRANT CRYPTS

INTRODUCTION

There are compelling epidemiological observations that suggest a role for selenium, a dietary trace element, in modulating gastrointestinal tract cancers (Clark et al., 1986). This observation is supported by the results of a ten-year clinical trial conducted by Clark et al (1996). These investigators evaluated the effects of high selenium containing yeast on various human cancers and showed significant reductions in the incidence of lung, colon, and prostate cancers (Clark et al., 1996). Preliminary chemical analysis of the high selenium containing yeast indicates that selenomethionine is a major constituent of selenized yeast. Thus, the components of selenized yeast responsible for the anticancer effects are of considerable interest.

Previous studies from our laboratory have shown that treatment of human colon cancer cells with selenomethionine resulted in apoptosis, mitotic alterations, and polyamine depletion (Redman et al., 1997). When

exogenous polyamines were added back to the selenomethionine-treated cells, the number of apoptotic cells was reduced to control levels (Redmanet al., 1997). This observation suggested that modulation of intracellular polyamine levels might play a role in the anticancer effects of selenomethionine.

The AOM model has been used successfully to test and evaluate mechanisms of action of chemopreventive agents, some of which are currently in clinical trials (Wargovich et al., 1990). Aberrant crypt foci (ACF) are easily recognizable precursors to colon cancer in carcinogen-treated rat colons (Bird et al., 1995). These precancerous lesions can be induced by colon carcinogens (McLellan et al., 1991), enhanced by tumor promoting diets (Tang et al., 1996), and modulated by inhibitors of carcinogenesis (Wargovich et al., 1992). ACF display genetic alterations and histological changes often seen in human colonic lesions (Stoperaet al., 1992), (Losi et al., 1996). The growth of ACF correlates with the adenocarcinoma yield (Magnuson et al., 1993). The present study investigates the hypothesis of whether dietary selenomethionine can suppress colonic polyamine levels in the AOM rat model of colon carcinogenesis.

MATERIALS AND METHODS

Animals, Diets, and Carcinogen Treatments

Three-week-old male F344 rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). The animals were acclimated for three weeks before exposure to azoxymethane. The carcinogen azoxymethane (AOM) was purchased from Sigma Chemical Co. (St. Louis, MO). The animal diets were obtained from Dyets, Inc. (Bethlehem, PA). Animals had access to food and water ad libidum. At 7 weeks of age, the animals in the control and experimental groups received AOM s.c. once per week for 2 weeks at a dose of 15 mg/kg body weight (Figure 3.1). At 8 weeks of age, groups of animals (10 rats/group) were fed the control diet or the experimental diets supplemented with 1 or 2 ppm of L-selenomethionine until the termination of the experiment. Animals were monitored daily for general health, and body weights were recorded every 2 weeks for the duration of the study. The experiment was terminated 16 weeks after the second AOM treatment, at which time all animals were euthanized by CO_2 asphyxiation. After blood was collected for measuring plasma selenium levels, the colons were resected and opened longitudinally, flushed with PBS and fixed flat in 10% buffered formalin.

Histopathologic Classification of Colon Lesions

All colon lesions were classified by a pathologist (Achyut K. Bhattacharyya, Pathology Department, University of Arizona Medical School). The fixed colons were stained with 0.2% methylene blue. The ACF throughout the entire colon were visualized using a light microscope (40X) for number and multiplicity. The criteria used to identify an aberrant crypt include: (a) increased size; (b) a thicker epithelial cell lining; and (c) an increased pericryptal zone relative to normal crypts. The microadenomas were visualized using a light microscope (40X) with H & E. The criteria that was used to define microadenomas included: (a) an increased size, (b) dysplasia, (c) an increased nuclear to cytoplasmic ratio, and (d) increased cellularity. Furthermore, crypts within microadenomas have an abnormal appearance and contain abnormal cells that do not produce mucin.

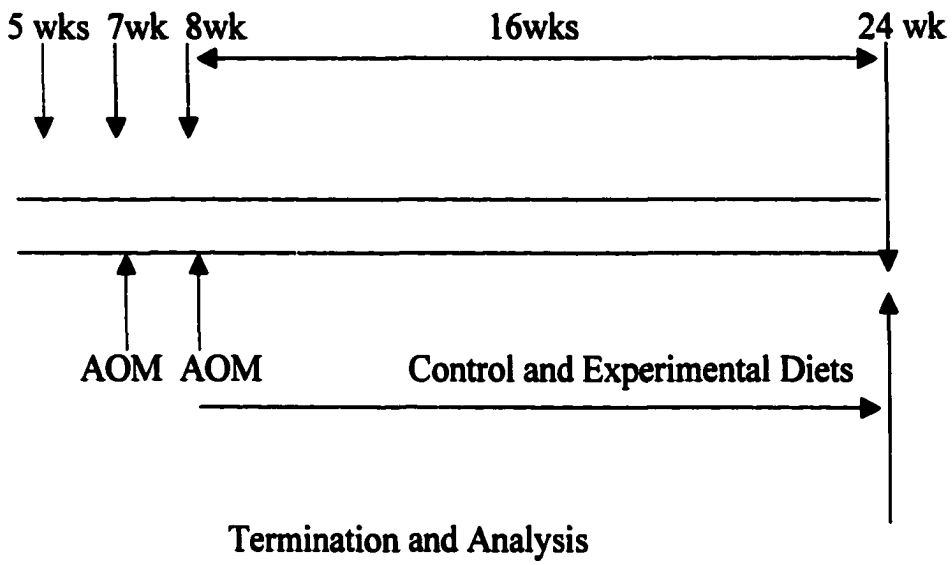


Figure 3.1 Experimental design of the chemopreventive study of dietary selenomethionine in F-344 rats.

Polyamine Analysis

A portion of flat colonic mucosal tissue was taken for intracellular polyamine analysis. Briefly, specimens were processed in 0.2 N perchloric acid ($HClO_4$) by homogenizing with a tight-fitting Teflon pestle homogenizer, and vortexed vigorously. These lysates were stored at 4 °C overnight, re-homogenized, and vortexed the following day. This treatment precipitates the majority of cell proteins, while free polyamines are soluble. The acid-soluble fractions were determined by high-performance liquid chromatography using the method of Seiler and Knodgen (1980). Polyamine measurements were normalized to protein content in the acid-

insoluble fraction. Protein levels were assessed using the bicinchoninic acid method, according to the manufacturer's instructions (Pierce Chemical Co, Rockford, IL).

Selenium Plasma Levels

In order to determine the selenium levels of the animals fed the different diets, plasma samples were collected from all the animals at the termination of the experiment. Selenium concentration in the plasma was determined by the fluorometric procedure of Spallholz et al.(Spallholz et al., 1978).

Liver Toxicity Assays

Liver toxicity was evaluated by histopathological examination and by alanine aminotransferase (ALT) activity. A separate group of non-initiated animals was administered control diet and Se-containing diets (1 ppm or 2 ppm) for 16 weeks analogous to the AOM-treated rats. After 16 weeks, these animals were sacrificed and blood and liver specimens collected. Livers were fixed in formalin, paraffin embedded, and stained with hematoxylin and eosin. Histopathological examination was performed to detect hepatic damage. Furthermore, plasma ALT levels were measured in

all the animals using an ALT reagent kit (Sigma) according to the manufacturer's instructions.

Statistical Analysis

The incidence of ACF was defined as the total number of foci containing aberrant crypts. Findings from Bird et al. (1995) indicate that the large ACF represent advancing preneoplastic states. Thus, the number of large ACF with 6 to 10 aberrant crypts per focus was analyzed for statistical significance using Poisson regression, a non-parametric statistical method. The Poisson regression method accounted for the number of ACF among the groups as well as the multiplicity of ACF. The Fisher exact test was used to analyze the association between microadenomas and the treatment groups.

A logarithmic transformation was applied to plasma and polyamine measurements to normalize the data. Analysis of variance (ANOVA) was used to determine the difference in selenium plasma levels and polyamine content between treatment groups. If a significant variation was detected, then a Tukey's multiple comparisons test was performed.

RESULTS

One of the concerns of using selenium derivatives as chemopreventive agents is the ability of the metabolites to get incorporated into proteins, thus

potentially accumulating in tissues at toxic levels. Liver toxicity assays and body weight measurements were performed to address this issue. There were no differences in feeding habits or average body weights between control and selenomethionine-treated animals (Figure 3.2). There were no changes in hepatic ALT levels nor were there any remarkable histopathological changes in the livers of animals fed selenomethionine relative to control diet animals (data not shown). Thus, selenomethionine given in the diet at 1 ppm or 2 ppm, for 14 weeks did not appear to have any adverse effects. The effects of dietary selenomethionine on the total number of ACF were evaluated as one of the endpoints in the study (Figure 3.3). It was found that 2 ppm selenomethionine reduced the incidence of ACF ($p= 0.001$), however, 1 ppm selenomethionine had no effect. Findings from Bird et al. (1995) indicate that large ACF with increasing crypt multiplicity represent advancing preneoplastic states. The multiplicity of ACF was defined as the number of aberrant crypts per focus (Birdet al., 1995). Large foci that contained 6-10 aberrant crypts were evaluated for multiplicity due their potential to progress to tumor formation (Birdet al., 1995). Lesions of these sizes were present in sufficient number for statistical analysis in all of the treatment groups. Selenomethionine at 1 ppm and 2 ppm reduced the

The Effects of Selenomethionine on Body Weights of Rats

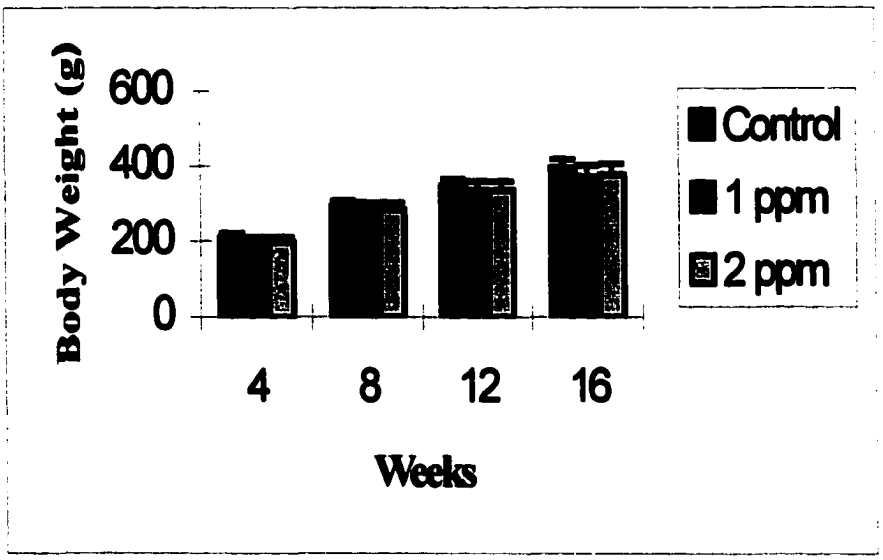


Figure 3.2 Body weights of rats fed selenomethionine. Mean ± S.E.

multiplicity of large ACF by about 2-fold compared to control ($p < 0.001$) (Table 3.1). The next endpoint evaluated was the effects of selenomethionine supplementation on microadenoma formation. A high percentage of animals receiving the control diet (90%) had microadenomas (Figure 3.3). In contrast, only 40% and 30% of animals given 1 or 2 ppm selenomethionine in the diet, respectively, developed microadenomas ($p = 0.06$, $p = 0.05$).

Putrescine, spermidine, and spermine levels were analyzed in mucosal tissue taken from the distal colon from control and selenomethionine-treated animals. The results of the HPLC analysis for polyamines are shown in Table 3.2. Although putrescine was reduced in the 1 ppm selenomethionine group ($p= 0.04$) relative to control, overall there were no significant differences in colonic tissue polyamine levels between the three treatment groups.

The last endpoint measured in the study was plasma selenium levels. Plasma selenium levels were lowest in control diet animals, intermediate in animals fed 1ppm selenomethionine, and highest in animals fed 2 ppm selenomethionine (Table 3.3). Plasma selenium levels in animals fed 2 ppm selenomethionine were significantly higher than that for the control group ($p=0.0006$). There was also a statistically significant difference between animals fed 2 ppm and 1 ppm of selenomethionine ($p=0.05$). Finally, there was no significant difference between 1 ppm and control.

The Effects of Dietary Selenomethionine on Aberrant Crypt Focus Incidence

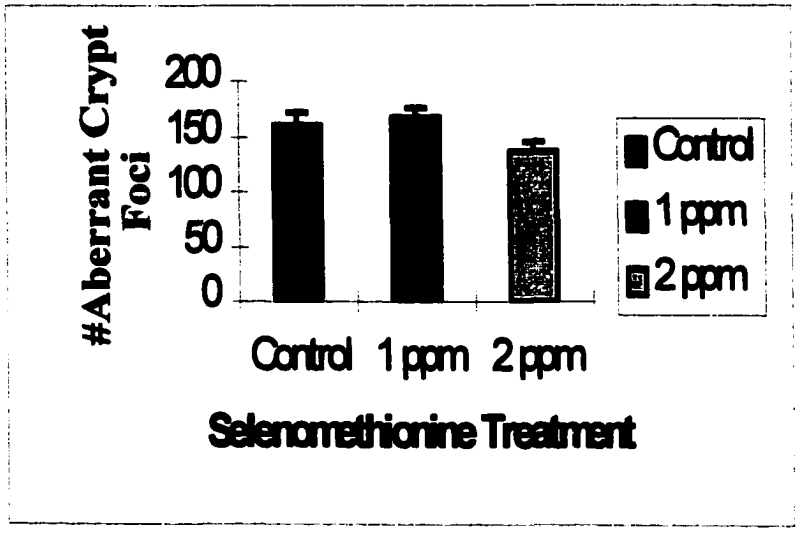


Figure 3.3 Incidence of ACFs with selenomethionine treatment. Mean ± S.E.

Table 3.1 The Effects of Dietary Selenomethionine on the Multiplicity of Aberrant Crypts [a]

Diet Groups	# Aberrant Crypts/Focus (6-10)
Control	6.5 ± 1.2
1ppm Se	$3..3 \pm 1.2^{b}$
2ppm Se	2.9 ± 0.85^{b}

[a]Mean ± S.E. (N = 10)
[b]Significantly different from control, $P \leq 0.001$ by ANOVA

The Effects of Dietary Selenomethionine on Microadenoma Development

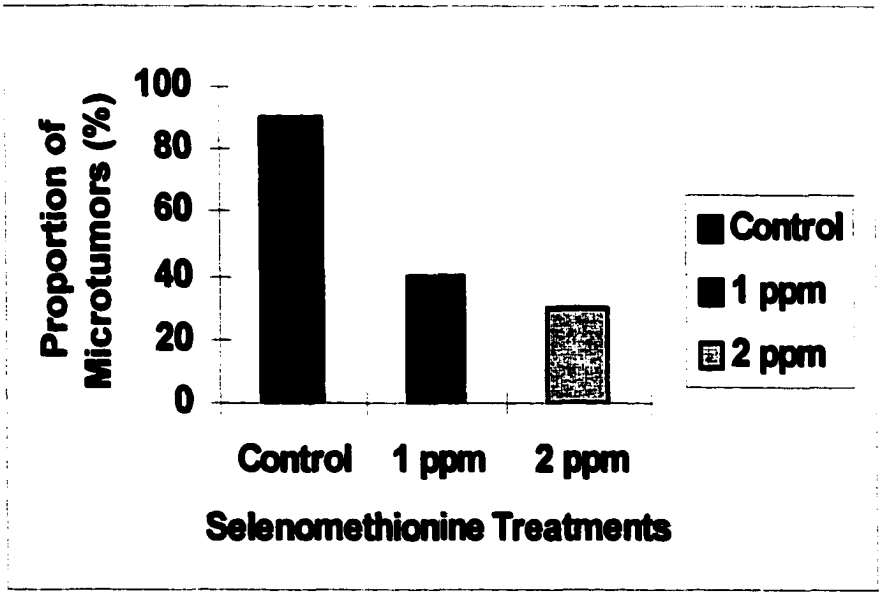


Figure 3.4 Effects of selenomethionine on development of microadenomas.

Table 3.2 Polyamine Content of Male F344 Rats Fed Dietary Selenomethionine.

		Polyamine content (pmole/ug protein)		
Exp. Groups	N	Putrescine	Spermidine	Spermine
Control	9	0.08 ± .18 [a]	0.43 ± .52	0.52 ± .67
1ppm	10	0.01 ± .01 [b]	0.53 ± .87	0.49 ± .77
2ppm	10	0.06 ± .08	0.62 ± .56	0.63 ± .65

[a] Mean ± SD
[b] Significantly different from the control diet group, P = 0.04
N= number of animals

Table 3.3 Plasma Selenium Levels of Male F344 Rats Fed Dietary Selenomethionine.

Experimental Groups	N[c]	Plasma levels (ng/mL)
Control	9	400 ± 90 [a]
1ppm	10	481 ± 87
2ppm	10	615 ± 132 [b]

[a] Mean ± SD
[b]Significantly different from the control diet and 1 ppm selenomethionine group, P = 0.00006
[c]N = number of animals per group

DISCUSSION

The results of a double-blinded, randomized, placebo-controlled trial by Clark and coworkers demonstrated, for the first time, that high selenium containing yeast, when administered as a supra-nutritional supplement, may be a very effective chemopreventive agent against several major human epithelial cancers (Clark et al., 1996). However, the mechanism by which Se-yeast exerts its anticancer effects remains to be established. This study evaluated the effects of selenomethionine (a major constituent of Se-yeast) on ACF growth characteristics, microadenoma development and intracellular polyamine levels.

Previous *in vitro* studies by our group suggest that selenomethionine-mediated growth inhibition may be associated with depletion of intracellular polyamine levels. A reduction in colon tissue polyamine levels was not found between control and seleno-methionine treated animals in the present study. Similar findings have been noted in studies using dimethylhydrazine-treated rats and selenite (McGarrity et al., 1993). Cellular polyamine levels are tightly controlled by a complex system of enzymes, allowing for the salvage, interconversion, and maintenance of polyamine pools (Pegget al., 1988). Also, depletion of polyamines in gastrointestinal tissues is especially

problematic since the gut has access to endogenous and exogenous sources of polyamines such as from luminal flora (Pegg et al., 1988). Thus, the anticancer effects of selenomethionine *in vivo* appear to be independent of polyamine depletion.

The results from the present study differ from observations from two recent studies which also evaluated the effects of dietary selenomethionine on colon carcinogenesis (Feng et al., 1999; Reddy et al., 2000). However, there are some important differences between these studies and our studies that may explain the conflicting findings. First, the type of carcinogen used and the timing of its administration differed in comparison to our studies. Fearon et al. (1999) used the carcinogen 3,2-dimethyl-4-aminobiphenyl, whereas, we used azoxymethane. Reddy's studies (2000) lasted for 52 weeks compared to our 16 weeks. Second, the dose of selenomethionine and the endpoints of these studies also differed. Reddy et al. (2000) used 10 and 15 ppm selenomethionine and focused on tumor counts. For our studies, we used 1 and 2 ppm selenomethionine and focused mainly on ACF due to length of the study. Finally, we utilized the active form of selenomethionine (i.e. L-enantiomer) whereas, Reddy et al. (2000) administered the less potent racemic mixture D,L-selenomethionine. Further studies may be necessary to

clarify these issues. Nevertheless, the results from the present study support recent human studies that suggest dietary selenium (in the form of high selenium-containing yeast) can reduce the incidence of benign and malignant colorectal lesions (Clark et al., 1996).

The mechanisms by which selenomethionine exerts its anticancer effects requires further investigation. Ganther et al. (1999) proposed that newly discovered selenoproteins such as mammalian thioredoxin reductase might play a functional role throughout the range of Se-mediated cancer prevention. The functional status of the thioredoxin/thioredoxin reductase system during *in vivo* chemoprevention with high selenium containing yeast components such as selenomethionine has not been established. Currently, research is ongoing to investigate this possibility. In any case, these results demonstrate that selenomethionine can inhibit the development of AOM-induced premalignant lesions through a polyamine-independent mechanism.

CHAPTER 4

THE EFFECTS OF SELENOMETHIONINE ON CYCLOOXYGENASES IN HUMAN COLON CANCER CELLS

INTRODUCTION

Being the second leading cause of cancer deaths, representing about 11%, colorectal cancer has become a major health concern in the United States (Ries et al., 1999) (Cancer Facts, 2000). The contribution thatnonsteroidal anti-inflammatory drugs (NSAIDs) has played in human colon cancer represents an important recent development in understanding the disease. Numerous reports, which include population-based studies, animal studies, and *in vitro* cell culture experiments, indicate that NSAIDs have anticancer effects (DuBois, 1985). Recent epidemiological studies document a 40-50% reduction in mortality from colorectal cancer in individuals taking NSAIDs (DuBois, 1997). The mechanism(s) responsible for these anticarcinogenic effects of NSAIDs are still unknown, but NSAIDs are known to inhibit the activity of the key enzymes, cyclooxygenases COX-1 and COX-2, required for the biosynthesis of various prostaglandins. Over-expression of COX-2 has been shown to play a role in rodent models of intestinal carcinogenesis (Oshima, 1996; Sheng, 1997), and treatment with selective COX-2 inhibitors

blocks the growth of various types of cancers, such as colon (Kawamori, 1998). The poor clinical prognosis in human lung and colon cancers has been associated with the presence of COX-2 (Achiwa, 1995; Sheehan, 1999). Also, COX-2 overexpression has been shown to occur in approximately 85-90% of human colon cancers (Eberhart et al., 1994). These findings have led to the development of selective COX-2 inhibitors to evaluate prevention and/or treatment of human cancers. One example of these new inhibitors is celecoxib (Celebrex) which has been shown to be effective in preventing the regression of adenomas in the adenomatous polyposis coli (Apc) mutant Min mouse model (Jacoby, 2001). It is being evaluated in combination with high selenium containing yeast in human clinical trials against polyp recurrence at the Arizona Cancer Center.

Selenomethionine is the primary form of selenium in the high selenized yeast being used in these studies. Selenium compounds have been shown to cause growth inhibitory effects *in vitro* (Redman et al, 1997), and inhibit and /or retard tumorigenesis in a variety of experimental animal models (Milner, 1985, El-Balyoumy, 1991, Combs, 1989). This leads to the question of the mechanism of action of selenium's anticancer effects. This series of experiments evaluates the hypothesis that selenomethionine may be

exerting its anticarcinogenic properties in part by modulating COX activity in colon cancer. The first experiments evaluated the effects of selenomethionine on the growth of several colon cancer cell lines that differ in COX-2 levels. The second group of experiments investigated the expression of COX protein levels in the selenomethionine-treated colon cancer cells. The third series of studies measured prostaglandin (PGE_2) levels in the selenomethionine-treated colon cancer cells. Finally, to gain insight into how selenomethionine affects COX-2, northern blot analysis was conducted.

MATERIALS AND METHODS

Cell Culture

The human colon cancer cell line HT-29 was cultured as described in Chapter 2. The human colon cancer cell line HCA-7 was a generous gift from the laboratory of Eugene Gerner, Ph.D. (Arizona Cancer Center, Tucson, AZ). This adenocarcinoma cell line, passages 15-35, was maintained in a monolayer culture in DMEM media (Mediatech, Inc., Herndon, VA) with 10% fetal bovine serum, 2 mM L-glutamine, 50 I.U./mL penicillin and 50 μg/mL streptomycin. The human colon cancer cell lines

Caco-2 (parentals) and Caco-2 (k-ras transfected) were also gifts from the laboratory of Eugene Gerner, Ph.D. These adenocarcinoma cell lines, passages 45-55, were maintained in a monolayer culture in MEM media (Mediatech, Inc., Herndon, VA) with 10% fetal bovine serum, 2 mM L-glutamine, 50 I.U./mL penicillin and 50 μg/mL streptomycin. The media was supplemented with 350 μg/mL of G418 Sulfate (Mediatech, Inc.). All cell lines were grown in a humidified incubator with 5% CO_2 at 37°C. Cell lines were monitored for the absence of mycoplasmas using the Mycoplasma Detection Kit (Boehringer Mannheim, Indianapolis, IN) by the Tissue Culture Core of the Arizona Cancer Center. Subculturing was done at subconfluent densities with a phosphate buffered saline solution of 0.25% trypsin (Gibco BRL).

Cell Treatments

The methodology and cell treatment with selenium is depicted graphically in Figure 2.0. All cell lines were maintained in T-175 flasks as previously described above. Cells were in log phase growth when they were passed into 100 mm tissue culture plates for treatments. To achieve cell growth in log phase, cells were passed two-three days prior to the first day of each experiment. Each cell line was plated at the following densities: HT-29 = 8

x 10^4 cells/plate, HCA-7 = 3 x 10^5 cells/plate, Caco-2 = 8 x 10^4 cells/plate. The concentrations of selenomethionine used to treat the cell lines were as follows:

HT-29 cell line: 32μM

Caco-2 cell lines: 32μM

HCA-7 cell line: 32μM, 60μM, 90μM (60μM was used for the PGE_2 experiments)

The selenomethionine was solubilized in media. Cells were collected at their respective time points for analysis every 2 days after treatment up to 6 days. For the cells that were not collected until later during the 6-day model, the old media was aspirated and provided with fresh media and treatment. There was a total volume of 10 mL in each plate. For harvesting of plates, media was aspirated and cells were enzymatically removed from the plate with a phosphate buffered saline solution of 0.25% Trypsin. To determine effects on growth, collected cells were counted using Trypan Blue (Sigma) exclusion. Trypan Blue was mixed in an equal volome with resuspended cells and viable cells were counted on a hemacytometer. Three different experiments were conducted to obtain average cell numbers for each time

point for the cell lines for the control and treated groups. The average cell numbers were plotted against time (days) in Excel.

Western Blot Analysis

Cell pellets from the HT-29 and HCA-7 colon cancer cells were collected and stored at –80°C. Cold lysis solution (50 mM Tris-HCl, pH 7.4), 250 mM sodium chloride, 0,5% NP-40, and 50 mM sodium flouride, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovandate , protease cocktail inhibitor) were added to the cells (between 300-600 uL) and vortexed. Then the pellets were sonicated for 1 second and kept on ice for 15 minutes. The samples were centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was transferred to an eppendorf tube and half of the original amount of lysis solution that was added earlier was added to the supernatant. Quantification of the proteins was performed using the Bicinochonic acid (BCA) Protein Assay (Pierce) and determined using a Biomek Plate Reader at 540 nm. Bovine serum albumin (BSA) was used for the standard. Cell lysates (45 μg) were run on 4- 20% pre-made acrylamide gel (Biorad) at 100V for 3-4hrs in electrode buffer (same as transfer buffer, but no methanol). The gel was washed in transfer buffer (25 mM tris base, 192 mM glycine, 0.1% sodium dodecyl sulfate (SDS), 15% methanol)

before being transferred overnight onto a PVDF membrane (Biorad). Before the transfer, the membrane was washed in 100% methanol until transparent for permeabilizing. The gel was transferred to the membrane overnight in transfer buffer in the cold room. After transferring, the membrane was incubated in 5% non-fat dry milk in PBS-Tween 20 (0.5%) for 1hr. The membrane was rinsed in PBS-T and then incubated with the primary antibody diluted in 1% BSA/PBS-T for 90 minutes. The concentrations of the primary antibody for COX-1 and COX-2 (Santa Cruz) were 1:200 and 1:5000, respectively. The concentration of the primary antibody of β-Actin (Sigma) was 1:500. Afterwards, the membrane was washed five times, once for 15 minutes and four times for 5 minutes in PBS-T. The membrane was then incubated with the secondary antibody diluted in 1% BSA/PBS-T for 60 minutes. The concentrations of the anti-goat secondary antibody were 1:1000 and 1:2000 for COX-1 and COX-2, respectively. The concentration of the anti-mouse secondary antibody for β-Actin was 1:15000. The same washing conditions as before were performed after the incubation. ECL chemilluminescence detection reagents (Amersham) were used to detect the presence of the antibody.

Northern Blot Analysis

RNA was isolated from cell pellets of the colon cancer cell lines (HT-29 and HCA-7) using the Rneasy Mini Kit (Qiagen) as described by the manufacturer. Cell pellets were lysed and homogenized and washed with ethanol. All incubations were carried out at room temperature. The resulting RNA product was eluted from the column using Rnase free water. The concentration of RNA was determined using a Beckman 640 spectrophotometer reading at an absorbance of 260 nm. Analysis of RNA expression was performed by running the RNA on a formaldehyde (37%)-denaturing agarose (1%) gel (Ultra Pure agarose (Gibco), 1X MOPS (Sigma)). Samples were concentrated with the use of a Speed-Vac for 20-30 minutes to get a final concentration of 10 or 15 μg/μL. The samples were brought up to a volume of 4 μL of RNase free water before adding 12 μL of loading buffer (500 μL formamide (Sigma), 70 μL 14.3X MOPS, 180 μL formaldehyde (Sigma), 10 μL ethidium bromide). The samples were heated at 65°C for 5 minutes, placed on ice for 10 minutes, and loaded into the lanes of the gel. The gel was run between 90-100V for about 3 hours in 1X MOPS buffer. A picture of the gel was taken using the lab polaroid before transferring the RNA from the gel to the membrane to insure equal

loading of the RNA and to assess RNA integrity. The gel was washed twice in 1X MOPS before being transferred onto a Genescreen hybridization nylon membrane (NEN). The membrane was wetted in water first, then in 20X SSC (3M sodium chloride (Mallinckrodt), 0.3M sodium citrate*$2H_20$ (Mallinckrodt), pH-7) for 5 minutes. The RNA samples were transferred to the membrane overnight in 20X SSC using a Vaccuum Blotter (Biorad). After the transfer was complete, the membrane was rinsed in 2X SSC and allowed to air dry. The membrane was crosslinked on the side that contained the RNA in an UV-crosslinker. The membrane was incubated in a pre-made prehybridization solution (UltraHyb) for at least an hour in Hybaid Rotating Oven at 55°C. The probes for the COX genes and GAPDH were made by adding 45 μL of TE buffer to 25 ng of COX cDNA (Oxford Biomedical Research) or 12.5 ng for GAPDH (made in the lab), boiling for 5 minutes, and keeping it on ice for 10 minutes. The DNA mixture was then added to RTS RadPrime DNA Labeling System Kit (Gibco BRL) and pipetted to resuspend. Afterwards, 5μL of ^{32}P-αdCTP (ICN) was added and mixed before incubated for 30 minutes at 37°C in a thermocycler (Perkin Elmer). Then, the mixture was added to Quick Spin G-50 Sephadex Columns-Fine (Boehringer Mannheim) and centrifuged for 4 minutes at

3000 rpm. The purified probe and herring sperm DNA (100 μg) was boiled for 10 minutes and kept on ice for 10 minutes before being added to the prehyb solution. The membrane was incubated overnight with the probe. The membrane was washed twice in 2X SSC, 2% SDS at 65°C for 45 minutes and twice in 0.1X SSC, 1% SDS at room temperature. The membrane was analyzed by phosphoimager analysis or autoradiography.

Prostaglandin E_2 Measurements

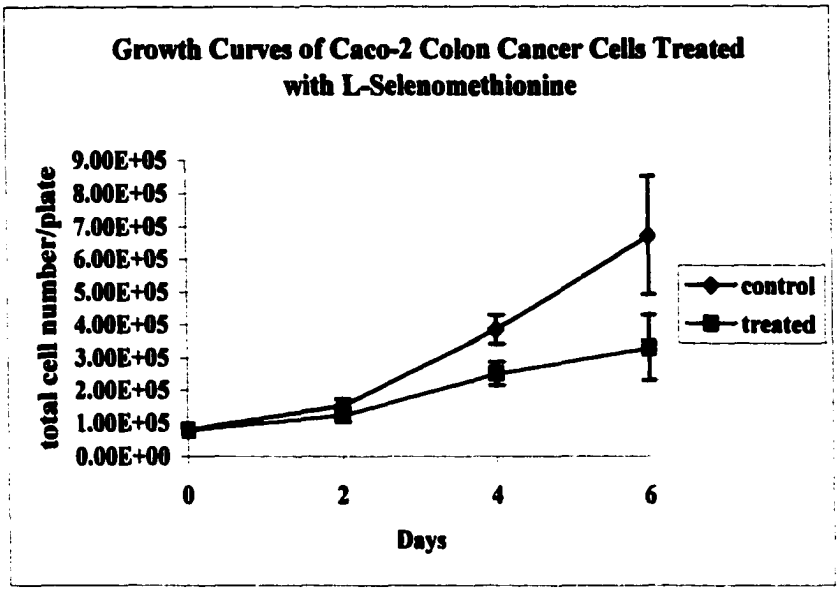
HT-29 and HCA-7 colon cancer cells were grown in the 6-day *in vitro* model as described earlier and treated with 32 μM and 60 μM seleno-L-methionine, respectively. Every 2 days the media was aspirated off and the cells were replenished in fresh media and drug containing no fetal bovine serum (FBS) and 15mM of arachidonic acid (Sigma) for an hour. Afterwards, 2mL of media was taken from the control and treated plates and stored in the -80°C until ready for analysis. Cell pellets were collected from the plates for protein analysis using the BCA protein assay for normalization. Prostaglandin E_2 (PGE_2) were measured using a Biotrak enzyme immunoassay (EIA) kit (Amersham). Protocol 1, the standard EIA procedure for measuring PGE_2 in cell culture supernatants, was performed

per manufacturer's instructions. The levels of PGE_2 was normalized to the respective protein concentrations and plotted on Excel.

RESULTS

Growth Inhibition in Human Colon Cancer Cells

Various colon cancer cell lines were used to evaluate effects of selenomethionine on growth in a 6-day *in vitro* repeated dose model (Table 4.1). Studies in Chapter 2 have previously shown growth inhibitory effects of selenomethionine on HT-29 colon cancer cells (Figure 2.2). Two other colon cancer cells used to evaluate selenomethionine's effects were HCA-7 and Caco-2. The IC_{50} for HT-29 cells (32 μM) was used to treat the Caco-2 and the Caco-60 (Caco-2 transfected with human k-ras oncogene). The transfection of the k-ras oncogene into the Caco-2 cell line results in the upregulation of COX-2 (Taylor et al., 2000). Treatment with selenomethionine was able to inhibit growth in both cell lines, with the Caco-2 being more sensitive than the Caco-60 (Figure 4.1). For the HCA-7 cell line, the concentrations of 32 μM, 60 μM, and 90 μM were used for treatments (32 μM not shown). With all the concentrations, dose-dependent



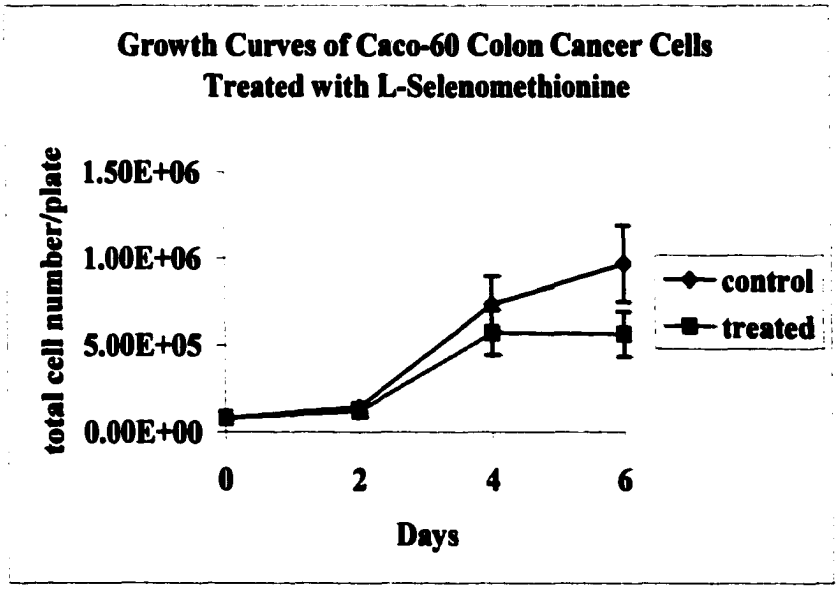


Figure 4.1 Growth curves of Caco-2 and the k-ras transfected Caco-60 treated with L-selenomethionine, respectively. Mean ± S.E. (n =3)

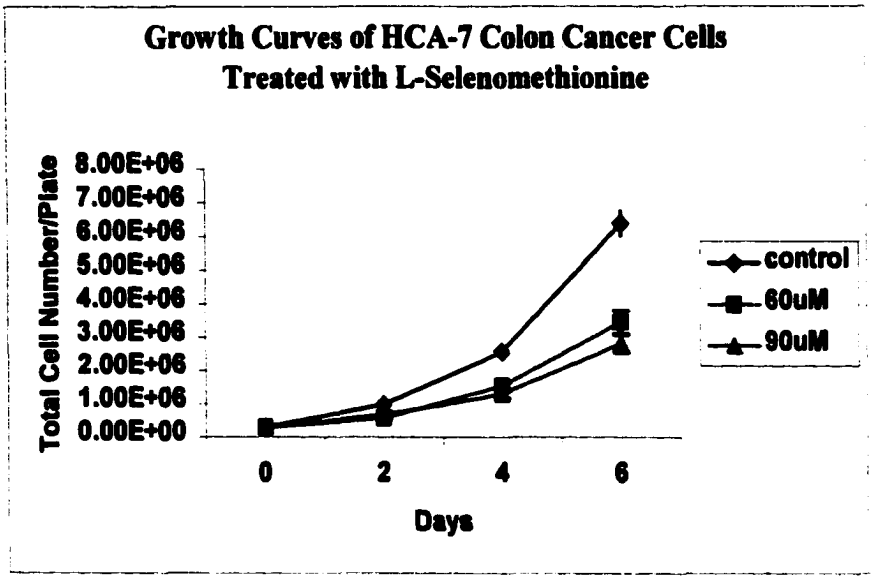


Figure 4.2 Growth curves of HCA-7 colon cancer cells treated with different concentrations of L-selenomethionine. 32μM was dose dependent and significant at day 6 (data not shown) Mean ± S.E. (n = 3)

growth inhibition in the HCA-7 was observed with increasing concentrations of selenomethionine (Figure 4.2). Collectively, these data indicate that selenomethionine inhibited the growth of several different colon cancer cell lines in a time and concentration-dependent manner.

Cyclooxygenase (COX) Protein Levels in Selenomethionine-Treated Cells

Next, we evaluated whether selenomethionine could affect COX-2 protein levels by western blot analysis. Proteins were isolated from cell

pellets from HT-29 and HCA-7 colon cancer cells grown during the 6-day *in vitro* model. In the HT-29 cells, there was no change in COX-1 protein levels at the different timepoints, but there was a decrease in the expression of COX-2 protein levels in the selenomethionine-treated cells at all timepoints (Figure 4.3). In the HCA-7 cells, there were no consistent changes in protein levels of COX-1 with the different doses of selenomethionine (Figure 4.4). However, with COX-2, there was a dose-dependent decrease in protein expression at the day 6 (Figure 4.4). Thus, selenomethionine specifically decreased COX-2, but not COX-1 protein levels in both HT-29 and HCA-7 colon cancer cell lines. Table 4.1 displays a summary of COX-2 expression and other genes found in three colon cancer cell lines.

Cyclooxygenase Protein Levels in Selenomethionine-Treated HT29 Colon Cancer Cells

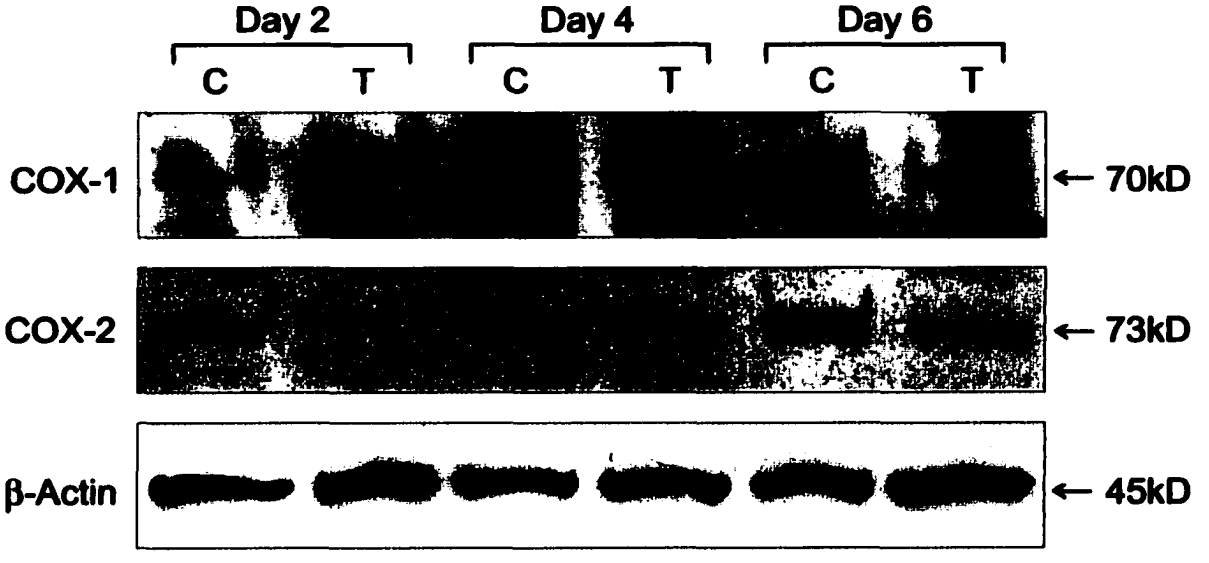


Figure 4.3

Cyclooxygenase Protein Levels in Selenomethionine-Treated HCA-7 Colon Cancer Cells

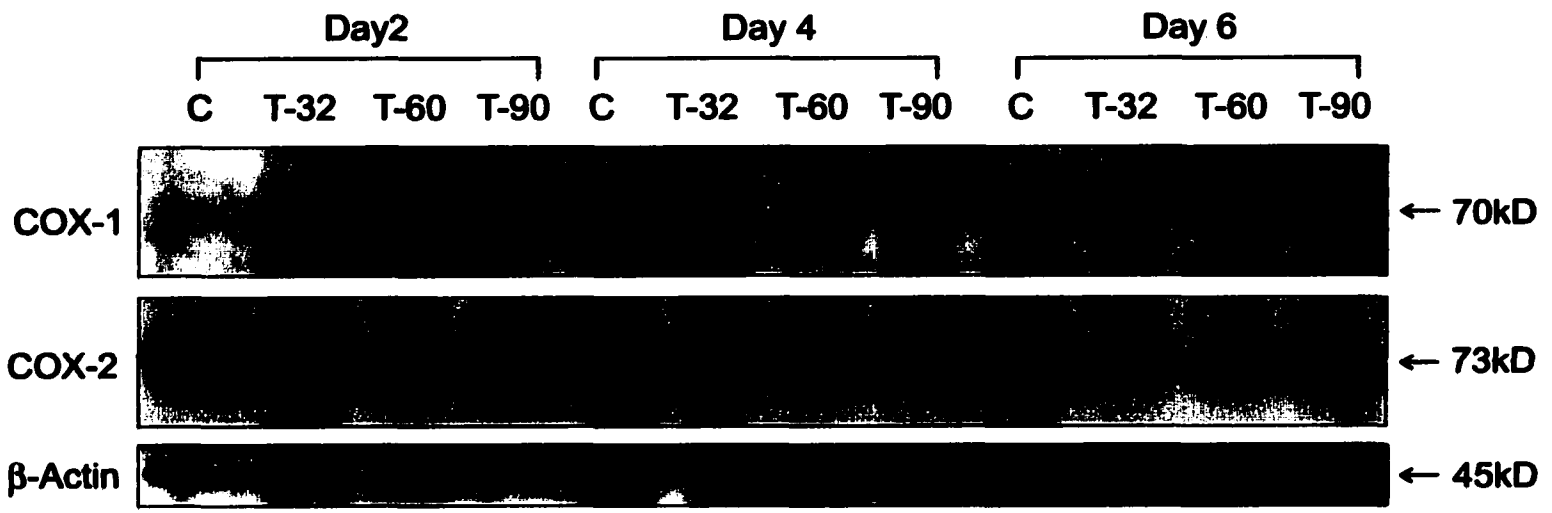


Figure 4.4

Table 4.1 Summary of some of the genes and genetic alterations involved in three colon cancer cell lines used in the study. +,++,+++ = increasing expression of COX-2, WT = wild type, MT = mutant type, ? = unknown

Cell Line	COX-2	Apc	p53	K-ras
HCA-7	+++	?	MT	MT
HT-29	+	MT	MT	WT
Caco-2	++	MT	MT	WT

Prostaglandin (PG) E_2 Measurements

Next, we measured levels of prostanglandin E_2 in serum-free media from the selenomethionine-treated HT-29 and HCA-7 cells that had been stimulated with arachidonic acid. The IC_{50} dose of 32 μM and 60 μM seleno-L-methionine were used to treat the HT-29 and HCA-7 cells, respectively. The media was collected from the 6-day *in vitro* model every 2 days. For HT-29 cells, there was a decrease in PGE_2 levels from selenomethionine-treated cells at day 6 (Figure 4.5). The decrease in PGE_2 occured at the same timepoint where growth inhibition was most significant. Similarly, there was a decrease in PGE_2 levels in the latter timepoints in the selenomethionine-treated HCA-7 cells, starting as early as day 4 (Figure 4.6).

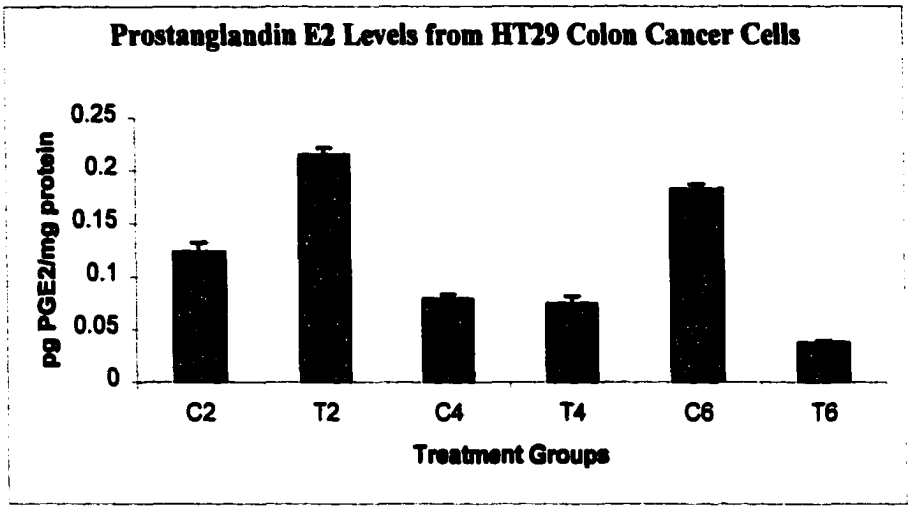


Figure 4.5 PGE_2 levels in HT-29 colon cancer cells after selenomethionine treatment. Cells were treated with the IC_{50}dose of 32 μM. Mean ± S.E.

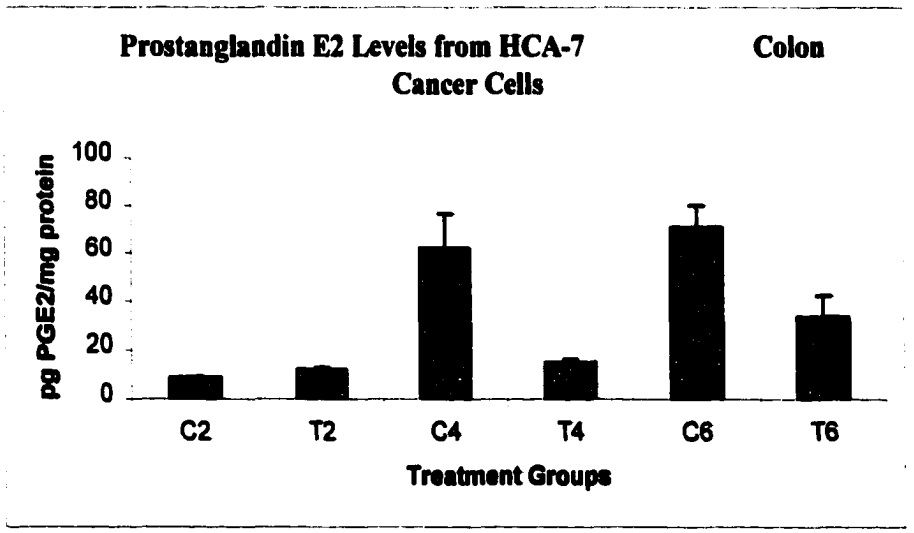


Figure 4.6 PGE_2 levels in HCA-7 colon cancer cells after selenomethionine treatment. The IC_{50} dose of 60 μM selenomethionine was used for treatments. Mean ± S.E.

The decrease in PGE_2 levels correlate well with the growth inhibition seen during the time course. Thus, selenomethionine treatment resulted in decreased PGE_2 levels in both HT-29 and HCA-7 cells.

Cyclooxygenase (COX) RNA levels in Selenomethionine-Treated Cells

Next, we performed northern blot analysis to gain insight into the mechanism by which selenomethionine decreases COX-2 protein. In the HT-29 cells, there was no consistent changes seen in RNA expression with COX-1 and COX-2 (Figure 4.7, 4.8). However, in the HCA-7 cells, there was both a dose-dependent and time-dependent decrease in COX-2 RNA expression at day 4 and 6 (Figure 4.9, 4.10). Consistent with the western blot analysis of COX-1 protein, there were no significant changes in COX-1 RNA. Analysis of GAPDH expression indicated equal loading of RNA samples.

Cyclooxygenase RNA Levels in Selenomethionine-Treated HT-29 Colon Cancer Cells

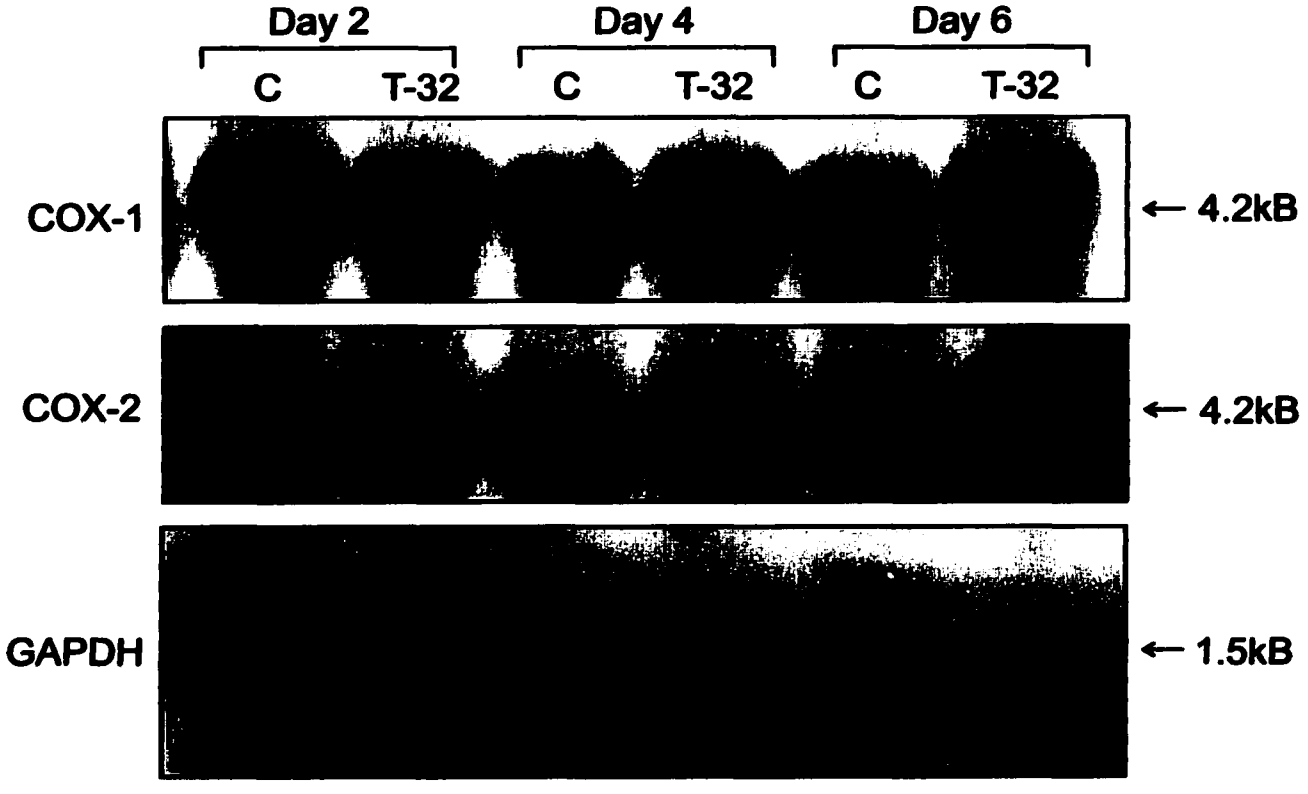


Figure 4.7

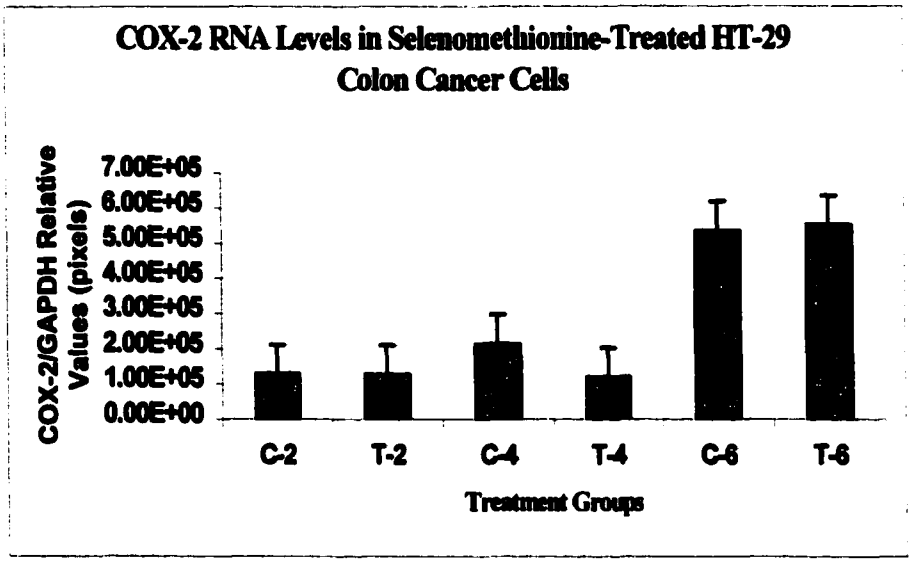


Figure 4.8 COX-2 RNA levels from selenomethionine-treated HT-29 colon cancer cells. COX-2 RNA was normalized to GAPDH. Mean ± S.E. (n = 3) Cells were treated with the IC50 of 32 μM selenomethionine

Cyclooxygenase RNA Levels in Selenomethionine-Treated HCA-7 Colon Cancer Cells

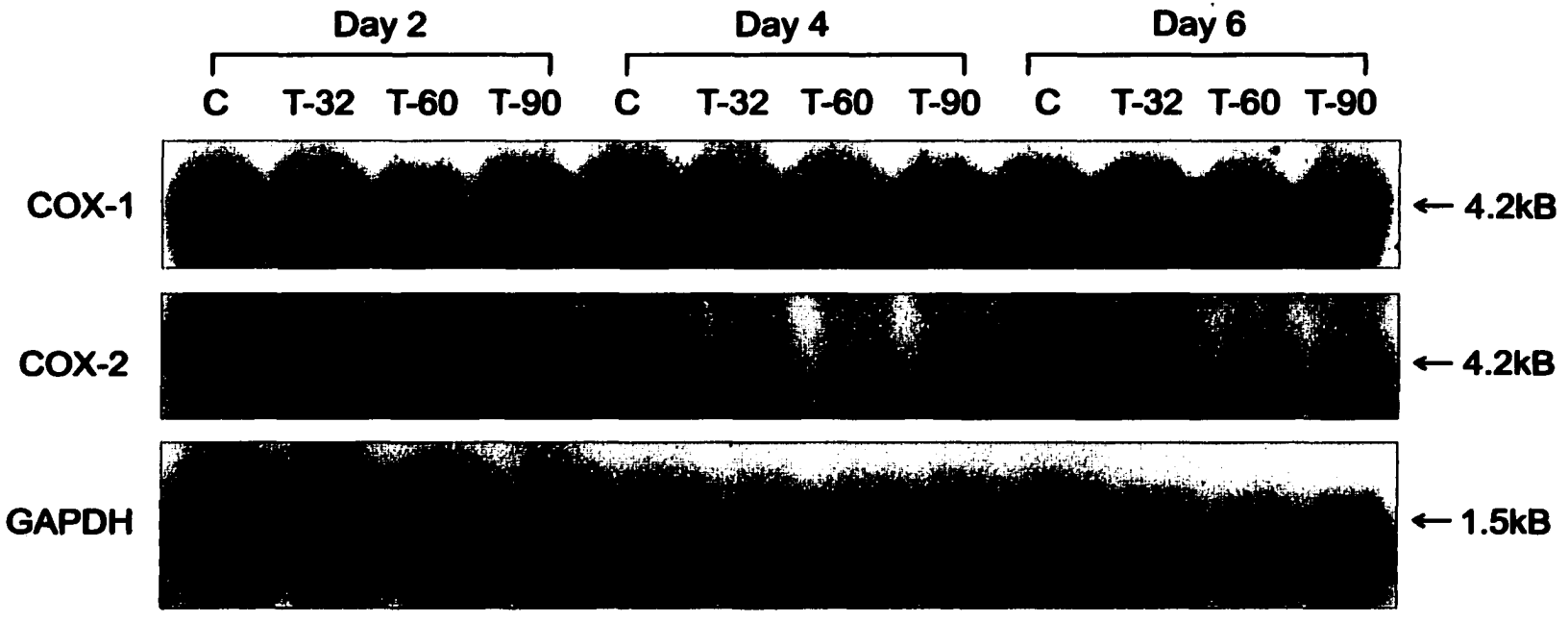


Figure 4.9

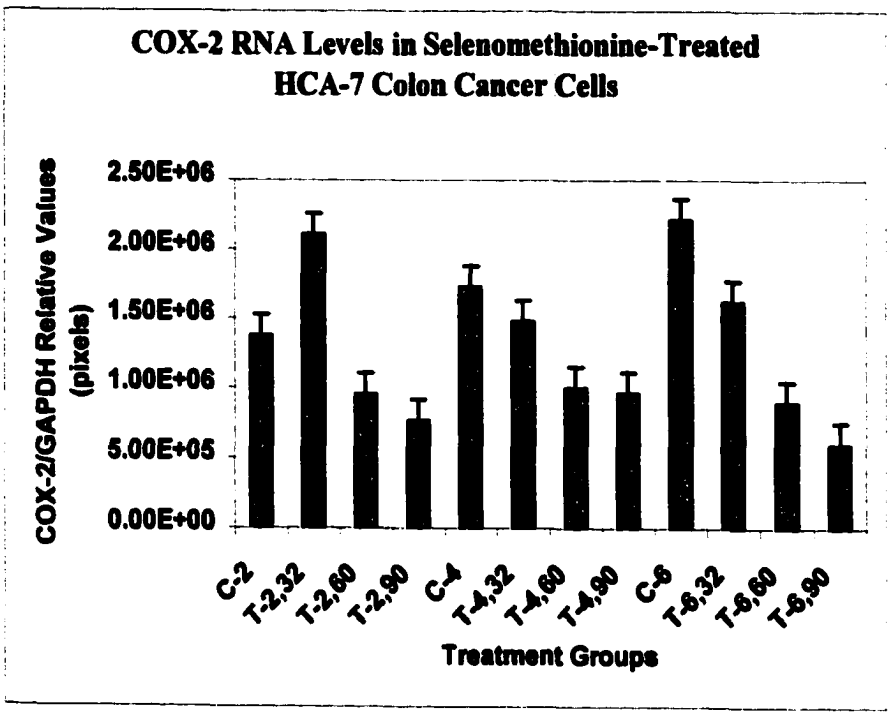


Figure 4.10 COX-2 RNA levels from selenomethionine-treated HCA-7 colon cancer cells. COX-2 RNA was normalized to GAPDH. Mean ± S.E. (n = 3)

DISCUSSION

These studies evaluated whether selenomethionine may be exerting its anticarcinogenic effects in part by modulating COX activity in colon cancer. The first series of experiments was to determine the effects of selenomethionine on growth of several colon cancer cell lines that differed in COX-2 protein levels. The four human colorectal carcinoma cell lines used for these studies were HCA-7, HT-29, Caco-2, and Caco-60. In order from most to least in reference to the amount of COX-2 protein, HCA-7 express the most COX-2, followed by Caco-60 and Caco-2, and HT-29 containing the least COX-2 (Shao et al., 2000 ; Taylor et al., 2000). All the cell lines were treated with seleno-L-methionine in the 6-day *in vitro* model to evaluate growth inhibitory effects. Growth inhibition was seen in all the cell lines with the HT-29 and Caco-2 cells being the most sensitive with significant decreases in growth as early as day 4. In the Caco-60 cells (Caco-2 transfected with k-ras), growth inhibition was not observed until day 6. This could be due to the transfected oncogene causing an increase of growth in this cell line and increased COX-2 levels (Taylor et al., 2000). For the HCA-7 cells that overexpresses COX-2, inhibition at 32 μM (the IC_{50} for HT-29 cells that was used for the other cell lines) was not significant

until day 6. Higher doses of selenomethionine showed a dose-response effect on growth inhibition for this particular cell line, including its IC_{50} of 60 μM. Overall, selenomethionine-induced growth inhibition was observed in all of the colon cancer cells that produced various amounts of COX-2 protein. This data demonstrates that selenomethionine can inhibit the growth of several different colon cancer cell lines *in vitro*.

The second objective of this study was to evaluate the protein expression of COX-2 in the selenomethionine-treated colon cancer cells. For these studies, HCA-7 and HT-29 cells were further analyzed. For the HT-29 cells, there was a decrease in COX-2 protein levels in the treatment groups at all 3 timpoints. COX-1 protein levels were analyzed as well, and there were no changes seen between control and treated samples. For the HCA-7 cells, there was a decrease at the day 6 timepoint in COX-2 protein levels in a dose-dependent manner. Analysis of COX-1 protein levels showed no consistent changes between control and treated samples. Selenomethionine effects were specific in modulating only COX-2 protein levels. This is important due to the role of COX-1 in various "housekeeping" functions such as maintaining normal gastrointestinal function (eg. mucus production) and other homeostatic roles within the

body. Toxicity (ulcers, bleeding, and renal impairment) attributed to COX-1 inhibition from NSAIDs have led to the discovery of specific COX-2 inhibitors, such as celecoxib which has been shown to prevent and regress adenomas in the min mouse model (Jacoby et al., 2000). Selenomethionine may serve as a potential agent in helping to inhibit COX-2 in colon cancer.

The third objective was to measure prostaglandin E_2 levels in the selenium-treated HT-29 and HCA-7 colon cancer cells. PGE_2 is the predominant prostaglandin produced by colon epithelial cells (Boughton-Smith et al, 1985) and has been shown to be upregulated in colorectal cancer (Earnest, D.L. et al, 1992). Media was taken from the various timepoints from the control and selenomethionine-treated samples after being stimulated with arachidonic acid and analyzed for extracellular PGE_2 levels. For the HT-29 cells, there was a significant decrease in PGE_2 levels with the selenomethionine treatment only at day 6. This could be explained by the decrease in protein levels of COX-2 seen at day 6. Day 4 showed no difference in levels between control and treated. In day 2, there was an induction of PGE_2 levels in the treated group. This induction could be one of the downstream effects of the cancer cell line trying to combat selenomethionine's growth inhibition. These PGE_2 results contradict studies

by Hsi et al. (2000) who provided evidence that the HT-29 cells had a catalytically inactive COX-2 protein. Our studies demonstrate that the HT-29's COX-2 protein is active due to the metabolism of arachidonic acid to prostanglandins in both control and treated cells, albeit, the amount ofPGE_2 produced in the cells is lower than other colon cancer cell lines. To further support our findings of selenomethionine having effects on COX-2, we used another colon cancer cell line, HCA-7, that has been used extensively for studying COX-2. For HCA-7 cells, there was a significant decrease in PGE_2 levels in the treated cells at days 4 and 6. There were no changes in prostaglandin levels at day 2. These results correlate well with the decrease seen in COX-2 protein levels at similar timepoints. The data present in this study helps to support the relevance of decreasing COX-2 and prostaglandins in preventing tumor formation.

The final objective of the study was to evaluate the RNA expression of COX-2 in the selenomethionine-treated cancer cells. Transcriptional regulation of COX-2 has been implicated in a number of studies in response to growth factors (Xie et al., 1996) and by signaling via oncogenic pathways in a variety of cells (Mestre et al., 1997). In the HT-29 cells, there was no significant difference in COX-2 RNA levels between control and treated

groups throughout the time-course of 6-days. Since there were significant decreases in COX-2 protein throughout the study, regulation of the COX-2 protein by selenomethionine must be occurring through other mechanisms. One potential pathway could be through increased degradation by the ubiquitin-proteasome pathway. Studies by Shao et al. (2000) showed that HT-29 cells have a high activity of ubiquitin proteolysis in comparison to other colon cancer cell lines such as HCA-7 cells. Secondly, due to the low amount of COX-2 in this cell line, changes in the RNA levels may be difficult to observe at the timepoints of interest. Earlier timepoints may need to be studied to notice RNA changes. On the other hand, In the HCA-7 cells, there were significant decreases in RNA levels in the treated groups throughout the study, with the most significant effects occurring at day 4 and 6. This correlated well with the decreased COX-2 protein expression and prostaglandin levels observed at the latter timepoints. This would suggest that the selenomethionine effects on COX-2 protein might be due to transcriptional regulation or message stability. Also, these results would suggest selenomethionine might suppress the growth of HCA-7 cells through a COX-2 prostaglandin-dependent mechanism. In summary, the data indicates that selenomethionine-induced growth inhibition may be a result of

modulation of COX-2 protein and prostaglandin levels. The modulation of COX-2 may be transcriptionally regulated in some colon cancers or may involve other mechanisms involving protein synthesis/degradation.

CHAPTER 5

DISCUSSION

The element selenium (Se) was recognized about four decades ago as being essential for the nutrition of animals and humans (Schwarz et., 1957). Since then, there have been numerous studies performed with this element to better understand its nutritional function, its many organic and inorganic metabolites, and its anticarcinogenic properties. Evidence from various epidemiological, animal, and human clinical trial studies has provided information supportive of selenium having potential anticancer efficacy. The most recent and promising piece of evidence that has led to the quest for understanding the mechanism of action of selenium was the nutritional prevention trial of cancer conducted by the late Larry Clark, Ph.D. (Clark et al., 1996). Selenium's ability to decrease the incidence of certain cancers, such as colon cancer, has provided the rationale for studying the role of selenium as a potential chemopreventive or chemotherapeutic agent against colon cancer. Since there are numerous forms of selenium, it is important that one does not generalize one selenium compound as having the same

qualities as another. The biological activity of each selenium compound is determined by its actual biological form. In the selenized yeast supplement used in the trial, analytical methods demonstrated that the predominant form of selenium was selenomethionine (70-80%) and the remainder being other selenopeptides. This discovery led to the decision to focus on this organic derviative of selenium for the studies presented here. Also, although all selenium compounds are toxic, the organic forms used in this study are much less toxic than the inorganic forms and are believed to be tolerated better by humans.

One major concern involving selenium in relationship to cancer prevention is understanding the mechanism of action that results in its anticancer properties. After the discovery of the first selenoprotein glutathione (GSH) peroxidase and its role of scavenging reactive oxygen species, it was expected that Se-dependent GSH peroxidase activity would provide a plausible mechanism for cancer prevention by selenium. However, it was found that GSH peroxidase activity was already at maximum levels in tissues of animals fed normal amounts of selenium (Yang et al., 1989). The enzyme levels did not change to any significant amounts as dietary selenium was increased to levels 10-fold higher which

are necessary to see chemopreventive effects in the animal models (Yang et al., 1989). Studies have shown that other selenoproteins reached a maximum level in tissues at nutritional selenium levels and excessive selenium intake may even have decreased activity (Behneet al., 1992). For example, levels of selenium that are antitumorigenic in animal models are greater than or equal to 1.5 ppm. The selenium levels required to support maximal activities of selenoproteins in animal models are less than or equal to 0.5 ppm. Concerning selenomethionine, studies have shown that this organic selenium derivative does not react with glutathione or generate superoxide (Yan et al, 1993). This supports the idea that there must be a unique mechanism other than selenoproteins to lead to the anticancer effects observed by this compound. Studies have shown that generation of a monomethylated form of selenium was a prominent feature of selenium compounds having good anticarcinogenic activity. Methylation is the best know fate of selenium and fully methylated metabolites are regarded as detoxified forms of selenium, which explains why these compounds may be preferred over the inorganic forms. Compounds such as selenomethionine and Se-methylselenocysteine, a monomethylated selenium derivative, are prodrugs that have to be metabolized to release the monomethylated

selenium moiety, which is believed to be the critical methylated metabolite needed for cancer prevention. The mechanism of action of these metabolites becomes the question that leads to the research presented in this paper.

In Chapter 2, the growth inhibitory effects of two organoselenium compounds, selenomethionine and Se-methylselenocysteine, were evaluated in lung, colon, and prostate cancer cell lines. These cancer typeshave been shown to be affected by selenium supplementation in the Clark et al., (1996) study. To begin to better study the duration of selenium exposure that humans would be exposed to as a potential anticancer agent, a 6-day*in vitro* model was created to elucidate the mechanism of action. The IC_{50}s for both compounds were used to evaluate effects on growth and the cell cycle. Both compounds were able to inhibit growth in a dose-dependent manner, but selenomethionine was found to be more potent. Studies by Ip et al. (2000) have shown Se-methylselenocysteine being more superior than selenomethionine in mammary cancer cells. Differences in the potency of different selenium compounds could be due to the presence or absence of various metabolizing enzymes in different cell types. Both of these selenium compounds are prodrugs that have to be metabolized to their active forms. Cells that are less sensitive to Se-methylselenocysteine may not have

the β-lyase enzyme needed to metabolize the compound. Alterations in the cell cycle in some of the cancer cells treated with the selenium compounds probably resulted in the inhibition of growth. Since the cells were asynchronous, some of the cells depending on what phase of the cell cycle, were probably more sensitive to selenium exposure than others, explaining the varying effects. Many *in vitro* studies involving selenium focused on earlier timepoints, but this study gives an indication of how cells may respond to continuous exposure of selenium over a longer period of time. These data demonstrate that selenomethionine and Se-methylselenocysteine may have inhibitory effects on growth over a period of 6 days by altering the progression of cells through the phases of the cell cycle. Since the cell cycle effects were not observed in all of the selenium-induced growth inhibition, other mechanisms must play a role. One potential mechanism could be modulating genes involved in growth and apoptosis. Selenium has been shown to induce the expression of various growtharrest and DNA-damage (gadd) genes in growth arrested cells (Kaeck et al., 1997). Further work needs to be done to better understand the gene regulation that controls the balance between proliferation and cell death that could potentially be influenced by various agents, such as selenium.

In Chapter 3, the purpose of the study was to evaluate the effects of selenomethionine on ACF growth characteristics, microadenoma development, and intracellular polyamine levels. A potential mechanism for selenomethionine-induced growth inhibition was hypothesized to be associated with depletion of intracellular polyamine levels. Polyamines are cations that are needed for growth and proliferation of cells. Due to the excessive growth of cancer cells, their need for polyamines is greater than normal cells. Since there is a common link between selenium metabolism and polyamine catabolism with the co-factor S-adenosylmethionine (SAM), it was suggested that selenium supplementation may prevent this co-factor from being available to help synthesize polyamines. This hypothesis was suported by previous studies by our group (Redman et al., 1997). The Azoxymethane (AOM) Rat Colon Carcinogenesis model is a well accepted model for studying colon cancer and has been used by many investigators for studying various chemopreventative agents as well as mechanisms involved in colon carcinogenesis. One of the concerns of using selenomethionine supplementation as a cancer intervention agent is the incorporation of this compound into various tissue proteins where it can be stored. Since selenomethionine can substitute for the amino acid

methionine, this uptake and storage could potentially lead to an excessive accumulation of selenium leading to toxicity. In this study, there was no evidence of toxicity from the selenium exposure from 1 and 2 ppm of selenomethionine. Other studies we conducted in the lab (unpublished data) have shown that 4 and 6 ppm selenomethionine had no toxic effects on the animals. This data suggests that high levels of selenomethionine may be better tolerated than once believed. This is supported by Schrauzer et al. (2000) who suggests the concern of incorporation of selenomethionine into body proteins and increasing selenium to toxic levels is not warranted because a steady state is established which would prevent uncontrolled accumulation of selenium. In this current study, there was a reduction in the size of the precursor lesions of aberrant crypt foci (ACFs) that has been correlated with the promotional stage of carcinogenesis. This reduction led to the decrease in microadenoma development in the treated groups. Concerning the potential mechanism of selenomethionine growth inhibition by polyamine depletion, there were no significant differences between control and treated animals in colonic polyamine levels. Other studies have shown the same result when other selenium compounds were used (McGarrity et al., 1993). Depletion of polyamines in gastrointestinal tissues

is especially problematic since the gut has access to endogenous and exogenous sources of polyamines such as the luminal flora (Pegg et al., 1988). Differences in polyamine levels in the selenomethionine-treated cells in the previous *in vitro* studies done by our lab group (Redman et al., 1997) may have been due to the isolated system of cells grown in tissue culture. Unlike the typical *in vivo* cellular environment, there are no endogenous or exogenous sources of polyamines for cells to obtain *in vitro*. Thus, the anticancer effects of selenomethionine *in vivo* appear to be independent of polyamine depletion. Overall results from this study indicate that selenomethionine is a good chemopreventative agent during the early development of colon cancer. A recent study conducted by Reddy et al. (2000) showed no effects with selenomethionine on the same colon carcinogenesis model that was used in this study. One major difference between the studies was in the isoform used to feed the animals. In Reddy's study, the D,L-isoform of selenomethionine was used, whereas, in the present study, the more potent L-isoform was used. A second difference was in the duration of the study. Reddy's study lasted for 52 weeks, whereas, our study was conducted for 16 weeks. In conclusion, selenomethionine's effects on well-developed colon carcinomas need to be

further investigated, but the results presented here demonstrate selenomethionine's efficacy on the early stages of colon carcinogenesis.

In Chapter 4, the purpose of the study was to evaluate the hypothesis that selenomethionine may be exerting its anticarcinogenic effects in part by modulating cyclooxygenase (COX) activity in colon cancer. This enzyme is responsible for metabolizing arachidonic acid to various prostaglandins. Two existing isoforms of this enzyme are COX-1 and COX-2, with the former's expression being constitutive and the latter being inducible. COX-2 has been shown to be upregulated in 85-90% of colorectal cancers (Eberhart et al., 1994). The first set of experiments was conducted to demonstrate that selenomethionine-induced growth inhibition could occur in various colon cancer cell lines that expressed varying amounts of COX-2 protein. Growth inhibition was also observed in HCA-7 cells, a cell line that constitutively express high levels of COX-2 proteins and HT-29 cells, a cell line that express low levels of COX-2 proteins. Growth inhibition was observed in the Caco-2 and the Caco-60s colon cancer cell lines, both intermediate COX-2 expressors. The latter cell line is the Caco-2 transfected with k-Ras, which has been shown to increase COX-2 expression (Taylor et al., 2000). The data proves that selenomethionine can affect a

number of colon cancer cell lines that expresses COX-2. The next set of experiments evaluated the expression of COX-2 protein between the HCA-7 and the HT-29 colon cancer cells. Selenomethionine treatment decreased COX-2 protein expression in both cell lines. This effect was specific to COX-2 with no changes observed with COX-1. This specificity is important due to the side effects that occur when COX-1 is inhibited by non-specific NSAIDs. Due to COX-1's role in homeostatic "housekeeping" functions, an anticancer agent that can specifically inhibit COX-2 would be very important in combating colon cancer.

The next set of experiments focused on the premise that decreased COX-2 protein would lead to a decreased production of prostaglandins. The prostaglandin of interest was PGE_2 due to it being the major prostaglandin produced by the colonic epithelium (Boughton-Smith et al., 1985). Selenomethionine treatment resulted in a decrease in the levels of PGE_2 in comparison to the control with both HT-29 and HCA-7 cell lines. The decreases were more evident in the HCA-7 cell line which expressed more COX-2 protein than the HT-29 cell line. The final set of experiments evaluated whether the decreased protein expression observed in the selenium-treated cells could be explained by changes in RNA levels. For the

HT-29 cell line, there were no significant changes in RNA levels between the control and treated throughout the 6-day time course. For the HCA-7 cell line, there was a dose-dependent decrease in RNA levels in the treatment groups. The differences in these two cell lines could suggest the potential of two different mechanisms being involved in the growth inhibition of colon cancer cells by selenomethionine, one pre-transcriptional and one post-transcriptional (Figure 5.1). One mechanism could involve decreasing COX-2 RNA levels. The decrease could be due to effects on mRNA stability or transcription activity. This would result in less COX-2 protein, leading to decreased prostaglandin levels such as PGE_2. This is the postulated mechanism of regulation in the HCA-7 colon cancer cell line. Future studies using nuclear run-on assays, RNA stability assays, and COX-2 promoter contructs should clarify this issue. Since there was no decrease in COX-2 RNA levels, but decreases in COX-2 protein and PGE_2 levels in the HT-29 colon cancer cells, another mechanism must be responsible for the growth inhibition. A potential mechanism for the decreased COX-2 protein expression in the selenium-treated cells could involve the ubiquitin-proteasome pathway. This pathway is the principal mechanism responsible

for the turnover of short lived proteins in eukaryotic cells and is a fundamental mechanism for cellular control. Studies by Shao et al. (2000) demonstrated that COX-2 protein was degraded through ubiquitin proteolysis. In the present study, selenomethionine may cause an induction in this pathway leading to an increase in degradation of the COX-2 protein in the HT-29 colon cancer cells. Future work utilizing specific proteosome inhibitors should help determine the importance of mechanism II and the anticancer effects of selenomethionine. Furthermore, the utilization of other colon cancer cell lines as well as *in vivo* models of colon carcinogenesis will aid in the delineation of which mechanism is most important for selenomethionine anticancer effects.

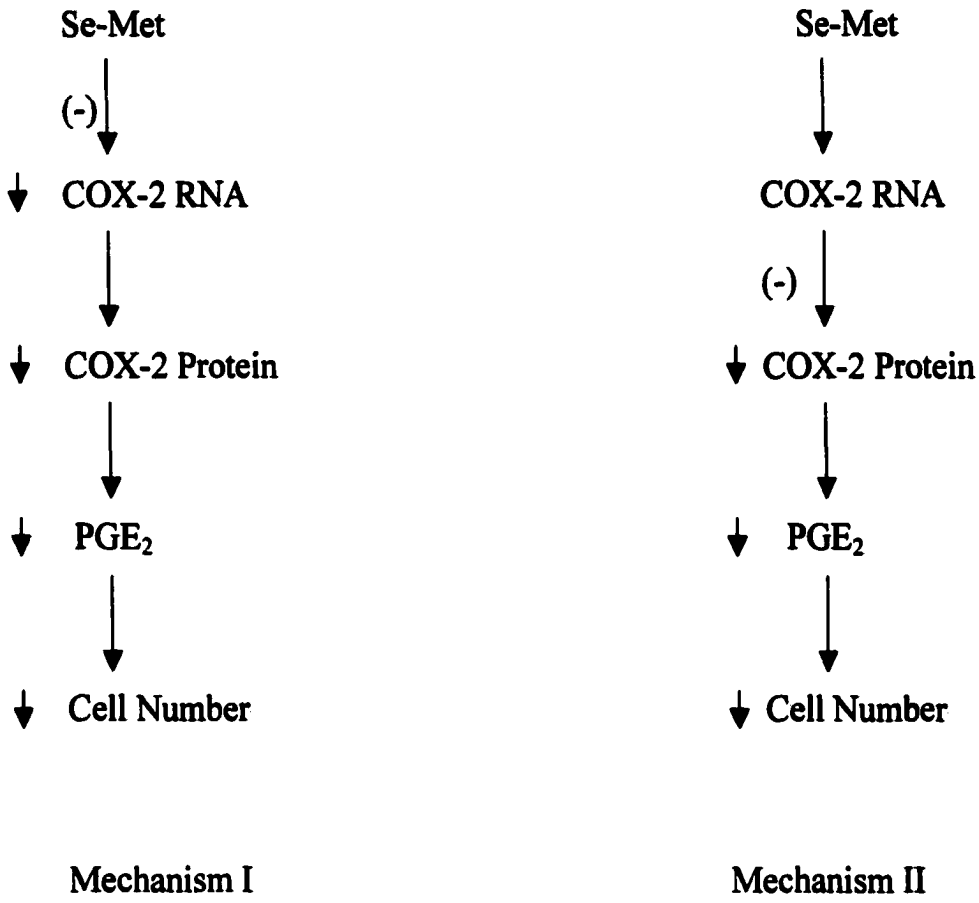


Figure 5.1: Proposed mechanisms of how selenomethionine may be having its antiproliferative effects on two colon cancer cell lines. Mechanism I represents the HCA-7 cell line where selenomethionine is having its initial effects transcriptionally or on mRNA stability, decreasing RNA expression. Mechanism II represents the HT-29 cell line where selenomethionine is having its initial effects post-transcriptionally, decreasing protein expression. The end result of both mechanisms is the decrease in cell proliferation of both colon cancer cells.

Although there seems to be two mechanisms that may explain the decrease in COX- protein, both result in the decrease of prostaglandins, specifically PGE_2. Prostaglandins have been shown to play roles in a wide spectrum of biological processes (DuBois et al., 1998). The mechanism of action of prostaglandins involves binding to two distinct receptors, oneof which is the nuclear hormone receptor peroxisome proliferator-activated receptor (PPAR) that is a ligand-activated transcription factor (Figure 5.2). Three distinct PPAR isoforms, α, δ, and γ, have been isolated and characterized (Wilson et al., 2000). Studies have shown that PPAR plays a role in colon cancer (Sarraf, P., et al., 1999). The other receptor that prostaglandins, specifically PGE_2, can bind to is EP (prostaglandin E) receptors. Several important actions of PGE_2 are exerted via each of the four PGE_2 receptors subtypes: EP_1, EP_2, EP_3, and EP_4. PGE_2 has been shown to participate in colon carcinogenesis via EP_1 (Ushikubiet al., 1995). One of the downstream effects of prostaglandins is increased cell proliferation. Selenomethionine-induced growth inhibition may be due to decreased COX-2 protein levels that lead to decreased PGE_2 levels.

Selenomethionine is currently being evaluated in clinical trials against prostate cancer and high selenium containing yeast (which is predominately

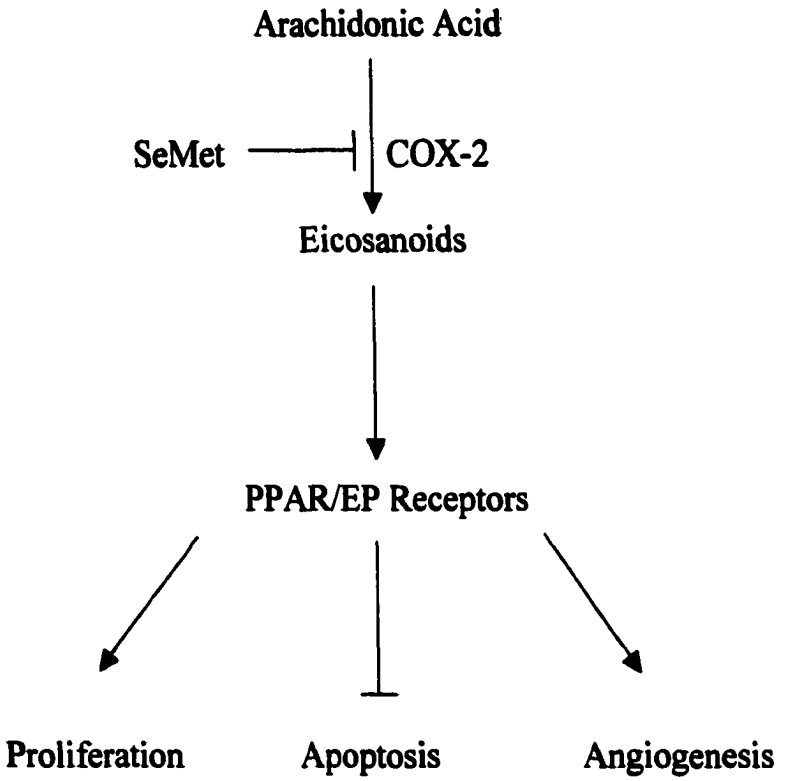


Figure 5.2: Potential mechanism of selenomethionine-induced growth inhibition on colon cancer.

selenomethionine) is being used in clinical intervention trials against colon polyp recurrence. Figure 5.3 illustrates a model adapted from Combs et at (1997) of how selenium may play a role in cancer prevention and treatment. Nutritional levels of selenium benefit the body by offering antioxidant protection, carcinogen metabolism, and immune system enhancement. In the prostate clinical trial, the maximal dose (3200 μg) of selenized yeast

given to patients achieved a blood serum concentration of 1012.4 ng/mL (1.01 μg/mL). This translates into a 5 μM concentration. Since

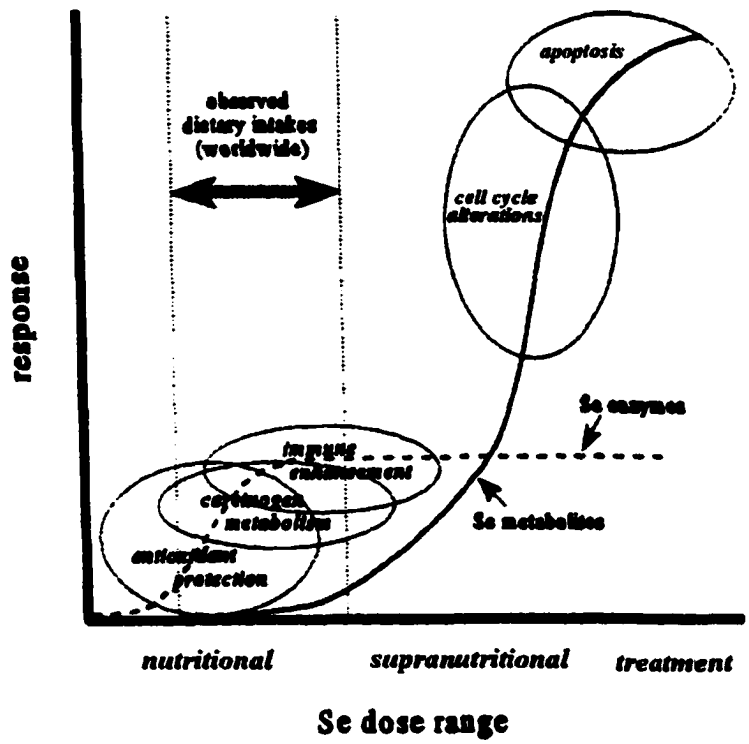


Figure 5.3 Model for the prevention and treatment of cancer using selenium. Adapted from Combs et al. (1997)

selenomethionine can get stored in tissue proteins, plasma Se concentrations are not a true indication of Se levels in the body. With this in consideration, Se levels are probably much higher and are in the supranutritional range in target tissues.

Collectively, these findings suggest that a constituent of selenized yeast, selenomethionine, impairs tumor cell growth through downstream targeting of COX-2. However, the signaling events underlying these anticancer effects are not clearly defined at the present time. In this set of experiments, selenomethionine was able to inhibit cell growth and induce some cell cycle alterations in a dose-dependent manner during continuous exposure. Also, the growth inhibition induced by selenomethionine may be by two distinct mechanisms, one involving transcriptional effects and the other may be by post-translational effects (ex. protein degradation). Selenomethionine reduced COX-2 protein levels, resulting in decreased prostaglandin levels. One of the many downstream effects of prostaglandins is proliferation, which was inhibited by selenomethionine. In conclusion, the findings presented in these studies give insight into the molecular mechanism by which dietary selenomethionine (and perhaps high selenium-containing yeast) exerts its anticancer effects. To our knowledge, this is some of the first work that demonstrate selenomethioninemodulating COX-2. Further work must be done to better understand how this modulation may be occurring, but the data presented here should suggest the importance of

continued use of selenomethionine as a tool to better understand the anticancer effects of selenium.

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