Chapter 10

Discussion
Cancer, a major killer throughout human history, changed its grasp as humankind advanced industrially and technologically. For all the time and money invested in breast cancer studies over the past two decades, epidemiologist have made little head way in explaining the long relentless increase in the incidence of this for decades over the past 50 years.

Chemoprevention is an attempt to use natural and synthetic compounds to intervene in early precancerous stages of carcinogenesis, before invasive disease begins. It basically involves a strategy to find out either component of food or pharmaceuticals. It started in mid 1950s when investigators first directed their knowledge of carcinogenesis toward search for substances that could inhibit tumor formation. This approach to cancer prevention was named chemoprevention in mid 1970s by Michael B. Sporn, an innovator in cancer prevention research. Since then hundreds of potential chemopreventive agents have been identified. However mechanism of their action is still not known.

Several studies have shown that stimulation of cell growth and development of various mammalian system involves a significant elevation of intracellular concentration of polyamines (91,208-210). Polyamines are small aliphatic amines that are essential for growth and differentiation (96,99). Polyamines (putrescine, spermidine and spermine) are found to be high in tumor cells and play a major role in control of breast cancer cell proliferation (177,179,180,367,368). Polyamine levels vary depending upon the stage of mammary gland development. From my findings it was observed that polyamine accumulation in mammary epithelium increases during estrous phase. The lactating mothers showed the highest levels of spermidine in their
mammary epithelium when compared to either virgin, pregnant, or weaned stage of mammary epithelium.

It was reported earlier that polyamine biosynthesis is regulated by estrogens (369). Polyamine are known to influence estrogen action at multiple levels. These include the association kinetics of estrogen receptor to specific DNA sequences (174), the synthesis of estradiol regulated cell cycle specific genes (175) as well as the synthesis and/or action of estradiol modulated growth factors (176,177,390). Based upon above reports, polyamine biosynthetic pathway was chosen for exploration of the mechanism of known chemomodulators of antitumor therapy in breast cancer.

During the mammary gland development and estrous cycle, it was found that levels of female sex hormones start to rise and reach maximum during estrous phase. Polyamine levels were also found to be the highest during estrous phase which probably explains that polyamines may be necessary mediators of hormone action (216). The decline of hormones during metaestrous and diestrous explains for the decline of polyamine content during these stages.

Inhibitor of the polyamine biosynthetic pathway, DFMO has been shown to exert a potent antiproliferative effect in several experimental breast cancer system \textit{in vitro} (367,368,371) and to a lesser extent \textit{in vivo} (183,372). DFMO is an enzyme activated irreversible inactivator of ODC (136), depletes the cells of polyamines and ODC in a dose dependent manner. DFMO decreases the synthesis of ODC protein also. The antiproliferative effect of DFMO was reversed by exogenous putrescine as expected. DFMO (0.1 mM) resulted in DNA fragmentation which may lead to
apoptosis as observed from morphological changes of DFMO treated cells. The nuclei were found to be either condensed or fragmented. DFMO has been found to be effective in some animal tumor models (136). When DFMO was administered well after initiation stage it greatly reduced tumor development in rodents treated with a wide variety of tumor promoters. ODC activity was enhanced by a wide variety of tumor promoters. ODC acts like an oncogene if its expression can be elevated to high enough levels with carcinogen treatment (388). These results and findings provide a strong rationale for possible use of DFMO as a chemopreventive agent.

The other enzyme responsible for the synthesis of spermidine and spermine is AdometDC. It is also an important target for the development of improved therapeutics modalities in breast cancer treatment. The inhibitor of AdometDC which is less toxic and more specific like CGP48664, is found to be growth inhibitory to breast cancer cells with IC_{50} value of 0.2 \mu M. CGP48664 induced expected increase in putrescine. Spermidine and spermine were reduced to 35\% and 40\% respectively of control. It resulted in decrease in decreases the S-phase fraction of the cell cycle and increase in percentage of cells in the G1 phase.

Several polyamine analogs such as bis(ethyl)polyamines have also been developed as antiproliferative agents (350,351). These inhibitors negatively regulate ODC and AdometDC activity (352). They are known to induce spermidine/spermine N^1-acetyl transferase (SSAT) activity (353,354). These analogs are known to inhibit cell growth through the depletion of intracellular polyamines completely (355). Bis(ethyl)polyamines, BE3333 and BE4444 have antiproliferative effect on MCF-7 cells with IC_{50} values of 0.4 \mu M and 250 \mu M respectively. BE3333 was found to be
a more potent antiproliferative agent than BE4444. Both these analogs resulted in inhibition of polyamines. Earlier reports have shown that accumulation of bis(ethyl)polyamine analogs is important for inhibition of cell growth by these analogs (357). It was not clear whether polyamine deficiency is required for growth inhibition which was not restored by provision of polyamines. The accumulated bis(ethyl)polyamine analogs are known to cause the inhibition of protein synthesis (358). The proteins involved in the ATP production were markedly decreased, followed by decrease of ATP content and swelling of mitochondria and depletion of mitochondrial DNA.

Effect of polyamine function in regulation of cell cycle traverse has been under investigation. Early studies used cell population synchronized by mitotic shake (389-392) or excess thymidine (393) and correlated changes in intracellular activities of polyamine biosynthetic enzymes and/or contents of the polyamines with progression through cell cycle phases. Some of these reports demonstrate two peaks of ODC activity and polyamines during progression through the cell cycle (389,390), with first peak occurring at or shortly before the onset of S phase and second occurring near G2-M border. With the advent of specific inhibitors of polyamine biosynthesis, it became possible to partially deplete cells of their polyamine contents and investigate the consequences of such treatment on cell cycle phase distribution and rate of cell cycle traverse (394-397). MCF-7 cells treated with 0.1 mM DFMO showed a slight increase in the number of cells in the G1 phase at 48h after treatment. After 24h it caused arrest of cells in G2/M phase and decreased the number of cells in S-phase and similar effects were observed again at 72h. Earlier reports on altered cell cycle phase distribution in cultured human carcinoma cells partially depleted of
polyamines by treatment of DFMO showed a partial response to cell cycle phase distribution at lowest concentration of DFMO (0.1 mM) after 48h of treatment. Higher concentration of DFMO resulted in marked increase in G1 fraction and decrease in S phase (236). S-adenosylmethionine decarboxylase (AdometDC) inhibitor, MGBG is reported to result in accumulation of cells in G1 (236,399,401) or S or G2 (402). The AdometDC inhibitor, CGP48664 and polyamine analogs, BE3333 and BE4444 which are found to result in partial polyamine depletion in MCF-7 breast cancer cell resulted in decrease in S-phase fraction and accumulation of cells in G1 phase. These studies indicate requirement for polyamines to maintain optimal rates of cell cycle traverse.

Chemomodulators used here to study mechanism of their action are taxol and antioxidants like selenium and retinoic acid. Taxol is a diterpene derived from western yew tree Taxus brevifolia and most widely used taxane at present. It promotes microtubule assembly and shifts equilibrium to stabilize the microtubules assembled form and thus prevents cell division (89). This study shows that taxol has antiproliferative effect with IC₅₀ value of 0.05 μM. But its cytotoxicity was reversed by 0.1 mM DFMO. The resistance to cytotoxic effect of taxol by DFMO was observed when it was given either together or before or after taxol treatment. DFMO effect was reversed by addition of putrescine which supports the possibility of a co-relationship between polyamine depletion and reduced cytocidal efficacy of taxol. Earlier work demonstrated striking effects on microtubules disaggregation when cellular polyamine levels were depleted in CHO cells (334). It is possible that polyamine depletion in MCF-7 cells may affect the structure of microtubules by involving the taxol binding site.
L-butathione sulfoximine (L-BSO) also protects the cells from taxol cytotoxicity. L-BSO depleted the cells of GSH, permitting oxidation of sulfahydrial group in tubulin which in turn prevents the polymerization of microtubules (339) the target site of taxol action. L-BSO resulted in partial reversal of G2/M block produced by taxol.

Selenium is a known chemopreventive agent. It acts mainly at promotional stage of carcinogenesis, a step which does not involve chemical carcinogen metabolism. Selenium is shown to have biphasic effect on cell proliferation, at lower concentration it stimulated the cell growth but higher concentrations were found to be inhibitory (248,249). Also higher concentration (10 µM) caused DNA fragmentation and apoptosis which was not observed at lower concentration (5 µM). Selenium inhibition of protein synthesis and cell proliferation could be due to stimulation by GSSG of protein kinase able to phosphorylate eukaryotic initiation factor 2 (eIF-2); thereby inactivating it (256). Selenium is essential part of the enzyme glutathione peroxidase (GSH-Px) and it increases the glutathione peroxidase activity and in turn glutathione (GSH). The increase in GSH-Px prevents formation of carcinogenic oxygen radicals and thereby defends organisms against the initiation of carcinogenic process. Biphasic effect of selenium is also observed on GSH and polyamine levels. It is well established that ornithine decarboxylase which catalyses the rate limiting step in polyamine biosynthetic pathway requires SH groups for its activity (251) and can be regulated by compounds that react with thiol groups (252). Thus inhibition of polyamine levels by selenium could be related to its effect on the glutathione content.
Another antioxidant used as a chemomodulator in this study was retinoic acid. Retinoids are important cellular, dietary factors that regulate differentiation and cellular growth. Exogenous administration of retinoids resulted in suppression of epithelial carcinogenesis (281, 282). In this work MCF-7 cells when subjected to different concentration of retinoic acid, showed that at low concentrations ($10^{-8}$ M to $10^{-6}$ M) it is stimulatory to growth and only at high concentrations $10^{-5}$ M and $10^{-4}$ M it is inhibitory, which supports earlier observation using trans and cis-retinoic acid in MCF-7 cell line (313). DNA fragmentation was also observed with growth inhibitory concentration but not with growth stimulatory concentration. Apoptosis may be the reason for growth inhibition caused by retinoic acid. Retinoids are also known to influence DNA synthesis (297, 298), cell division (299), RNA synthesis (300), post translational glycosylation of protein (302), lysosomal membrane stability (303) agglutination (304) and adhesive properties of transformed cells and stimulation of gap junction (305). Retinoids are also known to control the expression of ornithine decarboxylase (306). The findings reported in this study are unique. The effect of $10^{-7}$ M RA which was stimulatory to growth resulted in significant increase in ODC activity and putrescine level over untreated control. However no changes in spermidine and spermine levels over controls was observed. The increase in ODC activity and putrescine content therefore supports the stimulatory growth effect by $10^{-7}$ M RA. In contrast in this experimental system it was observed that $10^{-5}$ M RA that resulted in inhibition of cell growth resulted in increase in ODC activity and putrescine levels in MCF-7 cells. However a significant inhibition of cellular content of spermidine or spermine was observed. In support of this contention it is obvious that at growth inhibitory concentration, RA is having an effect on polyamines and ODC activity similar to that caused by AdometDC inhibitors like MGBG or
CGP48664 (314). It is a well known phenomenon that AdometDC inhibitors result in increase in ODC activity and putrescine levels and growth inhibitory effect is due to inhibition of spermidine and spermine content (314). Cell cycle analysis showed that retinoic acid inhibition of MCF-7 cell growth occurs through induction of G1 arrest with concomitant reduction in the proportion of cells in G2/M phase. No significant alteration in S phase was observed. Lower concentration of retinoic acid (10^-7 M) which was growth stimulatory resulted in an increase in the percentage of cells in S phase. Earlier reports have shown that retinoic acid inhibits cyclin-B and CDC2 expression, which are possibly responsible for the reduction of G2/M portion of cells (315). Retinoic acid is also shown to have marked effect on some of the key cell cycle regulatory proteins in MCF-7 cells (315). Cyclin D3 and CDK4 are likely the early targets of retinoic acids followed by reduced pRb expression and phosphorylation as well inhibition of e2F1 transcription factor which controls progression from G1 to S phase (287).

Adriamycin, an antibiotic of anthracyclin group, has been reported to be an effective growth inhibitor of several human tumors as well as leukemic cells. The present study shows the effect of adriamycin on human breast cancer cell line. Cells were found to be more sensitive to adriamycin induced cell kill during S phase and they were arrested in G2/M phase. The mechanism by which adriamycin exerts its antitumor cytocidal effects remains controversial (360,362-365). Among factors thought to contribute are inhibition of DNA and/or RNA synthesis arising from intercalation of the drug (360-362), DNA strand breakage resulting from peroxide and/or radicals generated from adriamycin semiquinone radicals (363) and DNA strand breaks resulting from effects of adriamycin on mammalian DNA topoisomerase II (364).
Induction of tumor incidence in Sprague dawley rats by NMU or DMBA shows that increase in latency period, incidence and tumor burden with NMU compared to that with DMBA is probably due to the fact that DMBA needs to be metabolized before it acts as carcinogen, as it is given through gastric intubation whereas NMU is injected directly into the circulatory system and does not require any activation. No difference of polyamine levels and ODC activity was observed between DMBA and NMU treated rats. However, there was a significant enhancement in polyamines levels when compared to normal mammary epithelial cells. In this study it was demonstrated that the dietary administration of selenium to rats during the initiation phase effectively blocks or suppresses NMU induced mammary carcinogenesis. Higher concentration, 5 ppm of selenium resulted greater reduction in tumor incidence when compared with 1ppm dose of selenium. Similar chemopreventive ability of dietary selenium during initiation and post-initiation phase of carcinogenesis was observed in mammary glands (272) and colon of rats (273). Feeding of lower amount of selenium during initiation phase significantly increased the polyamine levels, however higher dose of selenium i.e 5 ppm resulted in much lower increase of polyamines compared with 1 ppm of selenium. These results are comparable to \textit{in vitro} data. Also sodium selenite feeding affected the content of GSH in mammary tumors. Alteration of GSH content in rat mammary tumor may contribute to chemopreventive activity of selenium that was observed in this study.

The retinoic acid administration to rats during post-carcinogenesis phase of NMU-induced mammary carcinogenesis, showed not only there is appreciable decline in the number of tumor bearing rats but also there is decline in the mean number of tumors per animal. Retinoids are known to exert their influence on DNA synthesis
(297,298), cell division (299), RNA synthesis (300), protein synthesis (301), post-translational glycosylation of proteins (302). However which of the above mentioned effect is a key to elucidation of retinoid action remains to be seen. Modulation of mammary carcinogenesis by hormonal alternation as achieved by surgical ablation of ovaries shows that combination with retinoic acid treatment is significantly more effective in reducing the total number of tumors per animals though no difference in tumor incidence was observed. Latency period was prolonged significantly with combined treatment. Earlier reports also shows that mammary carcinogenesis is known to be modulated by hormonal alteration (320,321). Combination of antiestrogen tamoxifen and retinoids resulted in reduced tumor incidence in carcinogen treated animals (321,322).

Estrogen, a steroid hormone is known to play an important role in the induction and progression of human breast cancer (223,224). The majority of patients with ER positive tumors respond to hormone therapy. Earlier studies showed that estrogen induced ODC activity (226-228) and polyamine pathways, is interlinked with estrogentic regulation of cell growth (229,230). Manni et al. (180,229) showed that polyamines play an essential role in estrogen action in colonogenicity of hormone responsive, N-nitrosomethylurea induced rat mammary tumors. This study further suggests that antiestrogen are able to reduce the serum induced increase in ODC and polyamine levels (243). The present work also shows that estradiol stimulation of growth of MCF-7 cells is inhibited by antiestrogen, tamoxifen. This inhibition involves the ability to suppress the polyamine biosynthesis. Tamoxifen in combination with DFMO showed additive inhibitory effect on MCF-7 cells growth which suggests the importance of combination chemotherapy with antiestrogen and DFMO. This
combination therapy may prove to be an effective way of treating mammary carcinogenesis.

A DFMO resistant cell line was raised to study the mechanism of drug resistance. DFMO-induced overexpression of ODC has been found to be predominantly due to gene expression (344, 386, 387). In this study I also found the amplified product of the target gene and its enzyme activity along with polyamine levels. Earlier work showed that comparison of human breast cancer specimens to normal breast tissue reveals more profound alteration in polyamine pathway than a selective increase in putrescine (384). These include the elevation of the cellular content of spermidine and spermine, acetylated polyamines and spermidine and spermine N\textsuperscript{1}-acetyl transferases (384).

Resistant cell line against DFMO seemed an excellent model to test the cytotoxicity of other potential inhibitors of wild type against this resistant. The results showed that adriamycin, CGP48664 and polyamine analogs BE3333 and BE4444 were equally effective against DFMO resistant MCF-7 cells. DFMO resistant cells gave an opportunity to exploit the multidirectional inhibitory approach in the polyamine biosynthetic pathway. It is interesting to note that if cells become resistant to one inhibitor of the pathway, other inhibitors of the pathway like AdometDC inhibitor CGP48664 and polyamine analogs can be employed in overcoming the resistance.

The results confirm that the polyamine biosynthetic pathway can be exploited successfully not only against the resistant cell line, but also suggests that if breast
cancer cells develop resistance to one of the inhibitors of the pathway, other targets in the pathway can be chosen for controlling the cancerous growth. Beside necessitating carefully planned combined chemotherapy to circumvent possible developing drug resistance it has been suggested that the gene amplification induced arrangements may play a role in progression of the malignant process (387). The polyamine biosynthetic pathway seems to play a major role in the mechanism of action of these chemodulators and more detailed studies could reveal changes that may be important not only to the metabolism of polyamines, but very nature of human malignancy in general.