Chapter 11

Conclusion
1. Polyamine accumulation in mammary epithelium increased during estrous cycle and lactating mothers showed the highest levels of spermidine in their mammary epithelium when compared to either virgin, pregnant or weaned mothers. Since female sex hormone levels are known to rise during estrous phase and also in lactating mother, this explains that polyamines are necessary mediators in hormone action.

2. Polyamines were found to play an essential role in the growth of NMU and DMBA induced mammary tumors as was observed by high levels of ODC activity and polyamine content. Human breast adenocarcinoma cell line MCF-7 has a very active polyamine biosynthetic pathway.

3. Comparison of the ability of two chemical carcinogens NMU and DMBA to induce mammary tumors in rats showed that NMU is more effective in tumor induction than DMBA as latency period (appearance of tumors) was more and tumor incidence was high as compared to DMBA. It may be because DMBA has to be metabolized before it act as a carcinogen.

4. The availability of specific inhibitors of ornithine decarboxylase, the first and the rate limiting enzyme in the polyamine biosynthetic pathway has been instrumental in establishing the significance of polyamines in cell growth and differentiation.

   a) DFMO, an enzyme activated irreversible inhibitor of ODC, had an antiproliferative effect on the growth of MCF-7 with an IC_{50} of 0.05 mM. It depleted the cells of polyamines and ODC in a dose
dependent manner. The antiproliferative effect of DFMO was reversed by exogenous putrescine (1.0 mM).

b) DEMO (0.1 mM) resulted in a slight increase in the number of cells in G1 phase at 48h after the treatment of MCF-7 cells. At 24h and 72h after the treatment it caused arrest of cells in G2/M phase and decreased the number of cells in S-phase.

c) DFMO resulted in 40% fragmentation of DNA of MCF-7 cells as identified by $[^{3}H]$thymidine incorporation.

5. Estradiol resulted in stimulation of growth of MCF-7 cells with corresponding increase in polyamine content. This stimulation is inhibited by an antiestrogen, tamoxifen. The inhibition involves the ability to suppress the polyamine biosynthesis. Tamoxifen in combination with DFMO showed additive inhibitory effect on MCF-7 cell growth. A combination chemotherapy may prove to be effective way of treating mammary carcinogenesis.

6. The other polyamine inhibitors used in this study as chemo-modulators against breast cancer are AdometDC inhibitor, CGP48664 and polyamine analogs, bis(ethyl)pentamines, BE3333 and BE4444. All these agents exerted a potent anti-proliferative effect on breast cancer cells. The present findings revealed that:

i) CGP48664 resulted in increase in putrescine levels but decrease in spermidine and spermine. BE3333 and BE4444 both caused decrease in putrescine, spermidine and spermine significantly.
ii) CGP48664, BE3333 and BE4444 caused accumulation of cells in G1 phase and decrease in S-phase fraction.

7. Adriamycin, an anthracyclin antibiotic, which is most widely used as an antitumor drug, exerted a potent antiproliferative effect on breast cancer cells. The growth inhibition of cells by adriamycin was not accompanied by any significant effect on polyamine levels. It resulted in increase in cells in G2/M and inhibition of percentage of cells in S-phase.

8. Selenium, a known chemopreventive agent has been shown to have antitumor activity both in vitro and in vivo. Selenium was found to play a major role in polyamine metabolism.

*In vitro:*

i) It has biphasic effect on cell growth. At lower concentration it is stimulatory to cell growth but at higher concentration it is inhibitory. Inhibition of cell growth by selenium could be due to DNA fragmentation and apoptosis.

ii) Selenium has a role in the regulation of polyamine biosynthesis and it is possible that regulation of polyamine biosynthesis by selenium could be due to action of selenium on thiol group which in turn is required for the activity of ornithine decarboxylase. Selenium leads to decrease in the percentage of cells in the S-phase and increase in G1 phase cells.
In vivo:

iii) It was observed that selenium delayed the tumor induction (latency period) and decreased the tumor incidence in NMU treated rats. Selenium (5 ppm) leads to decrease in polyamine levels in NMU-induced tumors but lower concentration (1 ppm) leads to increase in polyamine levels. Selenium also caused increase in glutathione content in mammary epithelium.

9. Retinoid, another chemopreventive agent tested here, is an important cellular, dietary factor that regulates differentiation and cellular growth. It has been an effective chemopreventive agent in chemically induced mammary carcinogenesis.

In vitro:

i) It showed a biphasic effect on cell growth of MCF-7 cell line. Retinoic acid concentration ranging from $10^{-8}$ M to $10^{-6}$ M caused increase in the cell growth and concentration ranging from $10^{-5}$ M to $10^{-4}$ M decreased the cell growth. Growth inhibitory concentration of retinoic acid caused DNA fragmentation. Combination of RA with DFMO did not show any enhanced inhibitory effect on growth of MCF-7 cells.

ii) A growth inhibitory concentration of retinoic acid resulted in an increase in ODC activity and putrescine levels but decrease in spermidine and spermine levels. RA growth inhibitory concentration
had an effect on polyamine and ODC activity similar to that caused by S-adenosylmethionine decarboxylase inhibitors.

iii) Effect on cell cycle showed that RA inhibition of MCF-7 cell growth occurs through induction of G1 arrest with concomitant reduction in proportion of cells in G2/M phase. No significant alteration in S-phase was observed.

*In vivo:*

i) RA delayed tumor induction in NMU treated rats and also reduced tumor incidence.

ii) It resulted in inhibition of polyamine levels in NMU-induced tumors.

iii) Mammary carcinogenesis is known to be modulated by hormonal alteration. Depletion of female sex hormone by surgical ablation of ovary (ovariectomy) also reduced the tumor incidence along with delay in tumor induction in NMU treated rats. Retinoic acid in ovariectomised rats further increased the latency period and decreased the tumor incidence. Polyamine levels in mammary epithelium of ovariectomized rats were found below the level of normal mammary epithelium. This again emphasizes the relationship of female sex hormone and polyamine levels.

10. Taxol, a diterpene, is one of the most promising anticancer agents developed during the past decade.
i) Taxol resulted in a dose dependent inhibition of cell growth with an IC\textsubscript{50} of 0.05 μM.

ii) Taxol cytotoxicity was reversed by addition of DFMO, added either before, after, or together with taxol. Depletion of polyamines seems to be the reason for the reversal of taxol cytotoxicity as the DFMO effect was reversed by addition of exogenous putrescine.

iii) A characteristic effect of taxol on cells was a blockage of cell cycle at G2/M. No alteration in cell cycle phase distribution in cultured human carcinoma cells was observed when DFMO was given along with taxol. Thus mechanism of protection against taxol provided by polyamine depletion is not due to changes in cell cycle.

iv) Taxol also caused DNA fragmentation leading to apoptosis. When DFMO and taxol were given together there was no fragmentation or apoptosis. Addition of putrescine to DFMO and taxol treated cells caused DNA fragmentations and apoptosis, indicating the involvement of polyamines in taxol action.

11. L-butathione sulfoximine (L-BSO) is known to deplete the cell of glutathione by inhibiting glutathione synthesis. The effect of L-BSO on MCF-7 cells showed that:

i) L-BSO depleted the cells of glutathione content and had a dose dependent inhibitory effect on cell growth.
ii) Treatment of MCF-7 cell line with L-BSO to deplete cellular GSH levels protected the cells from cytotoxicity caused by taxol. Mechanism of protection against taxol provided by GSH depletion could be partly due to change in the cell cycle pattern.

12. The last part of this study was to develop a resistant cell line against one of the inhibitors of polyamine biosynthetic pathway, DFMO. DFMO is a mechanism based irreversible inhibitor of ODC, the first rate limiting enzyme of polyamine biosynthesis.

i) Resistance to DFMO led to increase in ODC activity with a concomitant amplification of ODC protein synthesis. This may have been a result of gene amplification. Increase in ODC activity was accompanied by corresponding increase in polyamine levels.

ii) The DFMO resistant cells were equally sensitive to specific AdometDC inhibitor, CGP48664 but were found resistant to another nonspecific AdometDC inhibitor MGBG, when compared to wild type cells. The resistant cell line was sensitive to polyamine analogs, BE3333 and BE4444. BE3333 was more potent compared to BE4444. DFMO resistant MCF-7 cell line MCF-7 was also sensitive to âdriamycin, an anthracycline antibiotic.

The present work shows the importance of polyamine biosynthetic pathway in breast carcinogenesis. The role of various chemomodulators and their possible mechanism of action in breast cancer, both in vitro and in vivo shows that polyamine
pathway is an important target for breast cancer therapy. Hence polyamine biosynthetic pathway can be exploited successfully for the treatment of breast cancer. Also, if at all the cancer cells develop resistance to one of the inhibitors in this pathway, other targets in the pathway can be chosen for controlling the cancerous growth. These studies also emphasize the importance of the carefully planned combined chemotherapy to circumvent possible development of drug resistance. Further studies could reveal changes that may be important not only to the metabolism of polyamine, but also the very nature of human malignancy.