CHAPTER 6

EFFECT OF ANTIESTROGEN AND ANTIPOLYAMINES IN 7, 12-DIMETHYLBENZANTHRACENE INDUCED MAMMARY TUMOR

6.1 INTRODUCTION

Extensive studies have been carried out on the usefulness of estrogen receptors in the management of human breast cancers. The measurement of estrogen receptors in the tumor cytosols was the first approach to this problem, and it allowed the selection of receptor negative patients who are likely to respond to hormonal manipulation (McGuire et al., 1975).

The determination of progesterone receptors (PR), which are known to be synthesized under the estrogen control improved the selection by detecting these ER+ patients whose tumors, despite the ER presence, suffer from a post receptorial damage in the hormone dependent mechanism (Horwitz et al., 1975). The experimental mammary tumors serve as valuable experimental tools in study of human breast cancer. These models have a number of features that make it particularly attractive to the experimental study, eg. tumor induction ease and reliability, organ site specificity, tumors of ductal origin, tumors of predominantly carcinomatous histopathological characteristics, tumors of varying growth factor and or hormone responsiveness. Majority of these experimental mammary tumors are hormone dependent and either regress or grow due to hormonal manipulation, thus showing close similarity to human breast tumors. The presence of estrogen receptors further confirm the role of hormones in the stimulation of the growth of such tumors. Other estrogen induced proteins
like peroxidase (DeSombre et al., 1975) glucose 6-phosphate dehydrogenase (Messeri et al., 1983), 52 K protein (Westley and Rochfort, 1979), Creatine Kinase (CK) (Reiss and Kaye, 1981) were investigated in the tumor tissue. It has been shown that estrogen induced product is an undoubted improvement in comparison to the simple estrogen receptor assay as well in better understanding of the hormonal regulation of breast cancers. However, there is no evidence that the induction of such product both in vitro or in vivo is followed by a full biological expression of the hormone action. The mechanism of estrogen action in breast cancer has been studied by the help of experimental mammary tumors. The experimental mammary tumors were induced by chemical carcinogens viz. N-methyl N-nitroso urea, 7,12 Dimethyl benzanthracene. 3-Methyl Cholanthrene (Sinha and Dao, 1974, Thompson and Meezer, 1983). The mechanisms by which steroid hormones regulate growth of human breast cancer have not been totally defined. Most in vitro studies have suggested that both estrogens and estrogen antagonists have a direct effect on proliferation of human breast cancer cells which contain estrogen receptor.

The use of the antiestrogen, Tamoxifen, is well established for the treatment of breast cancer both in advanced disease and in adjuvant treatment of primary breast cancer. Studies on the mechanism of action of antiestrogens suggest that their action in the most part, is through their interaction with ER (Jordan, 1987). The affinity of different antiestrogens for ER correlates well with their potency to cell growth. And once the antiestrogen is bound to ER, this complex is unable to carry out the functions of ER. Since ER is known to regulate gene expression at the transcriptional level, the mechanism of action of antiestrogens might involve the inhibition of estrogen induced transcription leading to inhibition of cell growth (Sutherland and Murphy, 1982). Earlier studies on the action of estrogens in target tissues showed that estrogens induce ornithine decarboxylase activity (Cohen et al., 1970) and it has been recently shown that the polyamine pathway is interlinked with estrogenic regulation of cell
Fig. 6.1 Pathway for biosynthesis and interconversion of polyamines.
growth. The inhibitory effect of antiestrogens on breast cancer cells could be reversed by the addition of polyamines. The biosynthetic pathway of polyamines is illustrated in Fig. 6.1. Ornithine is formed by the action of ornithine decarboxylase from arginine. Putrescine is converted into spermidine by the spermidine synthase. A second aminopropyl transferase termed spermine synthase adds an additional propylamine moiety to spermidine, forming spermine. The source of these propylamine groups is decarboxylated S-adenosylmethionine (Ado Met) which is produced by the action of S-adenosyl methionine decarboxylase (AdoMet DC). The other product of the aminopropyl transferase reactions is 5'-methylthioadenosine (MTA). The polyamine biosynthetic pathway has been studied extensively with the antipolyamines (Sunkara et al. 1987). It has well established that some macromolecular processes associated with abnormal and normal cellular growth and multiplication can be slowed by sufficient depletion of the polyamines, putrescine and spermidine (Pegg and McCann 1982). This can be achieved by DFMO, a specific enzyme activated irreversible inhibitor of ODC, the rate limiting enzyme of the polyamine biosynthetic pathway. Bartholeyns (1983). (2R, 5R), 6-heptyne 2,5 diamine was synthesized and shown to be specific enzyme activated irreversible inhibitor of ODC (Danzin et al., 1983). These two antipolyamines have been extensively studied in in vivo and in vitro experimental models.

This chapter deals with the effect of antiestrogen (TAM) and antipolyamines (DFMO & MAP) on the hormonal regulation of breast cancer cells has been studied using 7.12 DMBA induced experimental mammary tumor.

6.2 MATERIALS
6.2.1 Animals

Inbred Virgin Wistar rats, 60-90 days old were used for this study.
6.2.2 Chemicals Required

7,12-Dimethylbenzanthracene (DMBA) was obtained as a gift item from Dr. Ercole Cavalieri, USA.

Tris, EDTA, Dithiothreitol (DTT), Dextran, Norit A, Diethylstilbestrol (DES), Brij 35 solution (30% W/V) were purchased from Sigma Chemical Co., USA.

Naphthalene and 1,4, dioxan (AR) from E. Merck.

PPO and POPOP were purchased from BARC, India. O-phthalaldehyde and 2-mercaptoethanol were obtained from M/s. Pierce, U.S.A. N,N-dimethylcyclohexylamine (DCA) was purchased from Fluka, Switzerland.

$^{17}$B (2,4,6,7) $^{3}$H-estradiol Specific activity (102 Ci/mmol) was a gift from Amersham International, U.K.

Oncomax containing Tamoxifen citrate U.S.P. equivalent to 10 mg Tamoxifen/tablet was purchased from TDPL, India.

Polyamine standards-Putrescine (PU) Spermidine (Spd) and Spermine (spm) were obtained as gift items from M/s. Fluka, Switzerland.

Antipolyamines - D.L.α-Difluoromethyl ornithine (MDL. 71.782) and (2R,5R)-6-heptyne-2,5-diamine (MDL 72.175) were received as gift items from Dr. C.D. Houldsworth, MERELL DOW RESEARCH INSTITUTE, FRANCE.
6.3 ANIMAL MAINTENANCE

The animals were housed in plastic cages containing paddy husk as bedding material to absorb the urine and moisture. Rats were fed with standard pellet diet and water, *ad libitum*.

6.4 MAMMARY TUMOR INDUCTION

**Air Pouch Technique**

Air pouch was induced in the rats according to the method of Udayachander *et al.*, (1987) 3-5 ml of air was drawn into a glass syringe of 5 ml capacity and needle was blocked with a rubber cork to render air tight. The whole set was autoclaved at 15 psi for 20 min to sterilize the air inside the syringe.

2-3 ml of sterile air was carefully injected subcutaneously first beneath the mammary fat pad so as to produce a pouch containing sterile air. The air inside the pouch was allowed to stabilize for a day, prior to the administration of carcinogen.

6.4.1 Carcinogen Administration

20 mg of 7, 12, DMBA was dissolved in 1 ml of sesame oil and vortexed to obtain an uniformly dispersed emulsion. This emulsion was injected into the air pouch using a 5 ml glass syringe fitted with 18 gauge needle.
6.4.2 Experimental Groups

Animals were arranged into various groups (6 animals per group)

- **Group I** - Animals received 7,12 DMBA alone
- **Group II** - Animals received 7,12 DMBA and antiestrogen Tamoxifen.
- **Group III** - Animals received 7,12 DMBA, antiestrogen Tamoxifen and antipolyamine α DFMO 0.25% 0.5% and 1%.
- **Group IV** - Animals received 7,12 DMBA, antiestrogen Tamoxifen and antipolyamines MAP (0.1% to 0.5%)
- **Group V** - Animals received no carcinogen

6.4.3 Antiestrogen Treatment

7,12 DMBA treated animals were administered tamoxifen at a dose of 0.5 mg per 0.5 ml sesame oil per rat (for 2 days).

6.4.4 Antipolyamines Treatment

0.25%, 0.5% and 1% D,L-α difluromethyl ornithine were dissolved in distilled water and administered to different groups of experimental animals.

0.1%-0.5% MAP were dissolved in distilled water and administered different groups of experimental animals.

6.4.5 Sacrificing Procedure

Animals were sacrificed at the end of experimental period by cervical dislocation and tissue specimens were processed for histopathological examination, estrogen receptor assay and polyamine estimation.
6.5 METHODS

Tissue specimens (Mammary gland and Tumor tissues) were used for the determination of ER assay. Tissue specimens mentioned above along with liver homogenates were subjected to polyamine estimation by HPLC analysis using fluorescence detection Precolumn derivatization with O-phthalaldehyde-2-mercaptoethanol (Skaaden and Greibrokk, 1982).

6.5.1 Preparation of Reagents

O-Phthalaldehyde-2-Mercaptoethanol solution

10 mg OPA was dissolved in 1 ml of absolute ethanol and 50 μl of 2 mercaptoethanol was added to the above solution. This solution was known as OPA-2 ME reagent.

Preparation of Boric acid buffer (0.4 M)

50 gm of boric acid was dissolved in 1 litre of distilled water the pH of the solution was adjusted with 2 M KOH to 10.8 3 ml of brij 35 (30% W/V) solution was added to the above solution and stored in dark bottles.

Preparation of Boric Acid Buffer-OPA-2ME reagent

9 parts of the borate buffer was added to 1 part of the OPA-mercaptoethanol solution and mixed in a vortex mixer for 30 seconds.
Polyamine Stock Solution

The polyamine stock solution was prepared by dissolving 10 mg each of putrescine, spermidine and spermine in 200 ml of 0.1 M HCl. This solution was stored in 20 ml aliquots at -20°C.

The stock solution was diluted with 0.1M HCl to obtain final concentrations of 100, 200, 300, 400 and 1000 pmol per 50 µl.

Preparation of Mobile Phase

0.1 M N,N-dimethylcyclohexylamine (DCA) and 0.2M phosphoric acid in 85% methanol.

Preparation of Samples

Tissue specimens were homogenized in TED buffer and centrifuged at 15,000 x g and the supernatant was used for the polyamine analysis (HPLC) and ER assays.

Preparation of standard polyamines

The different concentrations of standard polyamines were prepared by diluting the stock solution with appropriate volumes of 0.1 M HCl.

Derivatization with OPA reagent

A 100 µl of the boric acid - OPA - Mercaptoethanol mixture was added to 30 µl of standard polymaine solution in a dark bottle and mixed well in a vortex mixer for 90-120 sec.
Estrogen-Receptor Assay (DCC Assay)

The method of McGuire et al., 1973 was followed:

6.6 DETERMINATION OF POLYAMINES BY PRE-COLUMN DERIVATIZATION WITH OPA-2 ME WITH REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Chromatograph - Operational Conditions

Column: RP-ODS - 10 μm column (C18) 250 x 4.0 mm
Mobile phase - 0.1 M N, N-dimethylcyclohexylamine (DCA) and 0.2 M phosphoric acid in 85% methanol
Flow rate - 1 ml/min
Oven temperature - 40-50°C
Detection - Fluorescence detection

6.7 RESULTS

Mammary tumor induction in female Wistar rats was achieved by injecting an emulsion containing 20 mg of 7,12 DMBA in sesame oil using a novel Air pouch technique. After the injection animals were grouped into different groups as mentioned above. The control group of animals developed mammary tumors in 120 days after the carcinogen injection Fig. 6.2. More than 70% of the tumors on histopathological examination revealed adenocarcinoma and 20% fibroadenoma and the remaining cases were of fibrosarcomas and other types Fig. 6.3, 6.4 and 6.5.

The estrogen receptor assay of control tumor with histology of adenocarcinoma was performed using DCC assay and ER content was found to be in the range of 90-760 fmol per mg cytosol protein, while the
Fig. 6.2 7,12 DMBA induced mammary tumor in female Wistar rat.
Fig. 6.3 Histopathology of 7,12 DMBA induced mammary tumor-Adeno carcinoma X 160.
Fig. 6.4 Histopathology of 7,12DMBA induced mammary tumor-Fibroadenoma X 160.
Fig. 6.5 Histopathology of 7,12DMBA induced mammary tumor-Malignant fibrous histiocytoma X 160.
fibroadenoma revealed 20-60 fmols/mg cytosol protein and fibrosarcoma had ER content of less than 15 fmol/mg cytosol protein. The cut off value for defining ER status has been kept as 10 fmol/mg cytosol protein and the tumors having more than 10 fmol/mg cytosol protein were considered as ER+ and those with less 10 fmol/mg were graded as ER-. ER values for the normal animals have been to be very low. When compared DMBA induced experimental animals. After 7,12 DMBA injection animals were grouped as mentioned earlier and received both antiestrogen and antipolyamines.

Animals received different concentrations of Tamoxifen and antipolyamines were shown in Fig. 6.6, 6.7, 6.8 and 6.9 as seen from these figures tumor induction was inhibited in experimental groups animals received anti estrogen and 0.5% MAP as well as in the case of animals treated with Tamoxifen and 1% DFMO. A small nodule was developed in experimental groups of animals which received either tamoxifen or antipolyamine alone. The estrogen receptor levels were found to be decreased in the case of animals treated with Tamoxifen and 0.5% MAP as well as in animals treated with Tamoxifen with 1% DFMO. In other groups a small nodule appeared after 150 days of antiestrogen and antipolyamines treatment. On histopathological examination of the small nodules revealed regenerative changes. However there has been no significant changes in the liver. The polyamine levels of different groups of animals were estimated by reverse phase high performance liquid chromatography using C18 (10 µm) ODS column. The chromatography pattern standard polyamines containing 500 pmol of putrescine, spermidine and spermine is illustrated in Fig. 6.10. Chromatography pattern of polyamine is illustrated in Fig. 6.11 from it can be seen that there has been noticable fall in the putrescine level. Polyamine levels of animals treated with Tamoxifen and 1% DFMO is illustrated in Fig. 6.12, from this chromatogram can be seen that were has been very low levels of putrescine when compared to animals treated with Tamoxifen with less percentage of polyamine (DFMO and MAP). From this study, the inhibitory effect of MAP and Tamoxifen was found to be significantly higher.
Fig. 6.6 Inhibitory effect of Tamoxifen on 7,12 DMBA induced mammary tumor.
Fig. 6.7 Effect of Tamoxifen and 1% DFMO on 7,12 DMBA induced mammary tumor.
Fig. 6.8 Effect of Tamoxifen and 0.5% MAP on 7,12 DMBA induced mammary tumor.
Fig. 6.9 Effect of Tamoxifen on 7,12 DMBA induced mammary tumor, with 0-3/+ MAP.
Discussion

Chemical carcinogenesis in the rat mammary gland has been well established that a single feeding dose of 20 i.p. DMBA as an emulsion induces mammary tumors within 150-180 days. The administration of DMBA as an emulsion is associated with tumor growth (Weinstein et al., 1979). However, this is not understood the growth behavior and biological process of tumors. Numerous workers have been studied extensively by numerous workers and nutritional manipulation (Spicer et al., 1987). The induction of tumors has been of interest to modulation through various dietary ingredients, particularly polyamines. In the case of animal groups treated with tamoxifen with high concentration, the tumors developed early in the experimental group. As seen from the results, there has been marked photodynamic effect has significant advantage of exposing the mammary gland to development of mammary tumors in the experimental group. In this study, a novel air pouch technique, which consists of both tamoxifen and polyamines, has been subjected to modulation through various dietary factors, which are also illustrated in Fig. 6.13.
Polyamines are found in all cells and are involved in differentiation (Pegg et al., 1982). The elevated polyamine levels are frequently found in the urine, serum and cerebrospinal fluid of cancer patients. It has been accepted that continued study of the clinical utility of polyamine assay and the use of polyamine biosynthesis inhibitors has lead to the development of improved assay methods for the determination of polyamine levels.

The use of precolumn derivatization procedures in estimation has been found to be superior to the conventional assay procedures. As seen from the chromatographic patterns and Table 6.13 among three polyamines, there has been no notable change in spermine and spermidine and significant changes in the case of putrescine thereby indicating the inhibition of tamoxifen. It has been observed from this study, the inhibition of MAP is found to be significantly more than DFMO.

6.7.2 Conclusion

The induction of rat mammary tumors in 60-90 days was achieved with a single dose of 20 mg of 7,12 DME oil and the induction of mammary tumors using Air pouch resulted in localized mammary tumors. The tumors were much shorter period of 120 days after 7,12 DMBA treatment was found that 70% of animals with tumors on histopathologic examination revealed adenocarcinoma with other mixed histologic types. As determined by the DCC assay revealed these tumors as independent. It is felt that this tumor model might serve as a model for the study of estrogen receptors. The use of antipolyamines like MAP at higher concentration in combination with DFMO as a useful model to study hormone dependency of breast cancer can reveal the molecular mechanisms involved in the hormonal polyamine biosynthetic pathway.