Chapter 1

Introduction: Review of Literature
1.0 **GENERAL**

Cancer is a widespread disease with multiple causes and multiple manifestations. Cancer arises when a normal cell breaks free from the normal restraints of growth and undergoes continuous proliferation; ultimately leading to the death of the individual due to a variety of causes. This disease is responsible for claiming millions of lives year after year. One in every five person dies of cancer in the world. According to the World Health Organisation (WHO) reports, cancer leads to the death of roughly six million people annually in the global scenario.

Cancer induction is a multi-step process. It starts when cells undergo continuous proliferation followed by migration of cells and invasion into different sites within the body. The later is known as metastasis. These cells then form masses of the tissue or tumors at distant sites in the body; a condition known as malignancy. However, other types of cancerous cells may remain localized at one site and these are known to cause benign tumors. Tumors composed of malignant cells become more and more aggressive over time and become lethal when they disrupt the tissue organ needed for survival of the organism as a whole. Of all types of cancer known, breast cancer is one of the most frequently diagnosed amongst women and second most frequent cause among cancer deaths after lung cancer.
2.0 EPIDEMIOLOGY

Amongst every eight women, one is diagnosed with breast cancer in their life time and one in every 28 dies of it (1). Internationally mortality rates per 10,000 women vary from less than 6 in Japan to almost 30 in England. Rates are low (less than 15) in Mexico, Costa Rica, Chile, Hongkong and Singapore and around 20 in Western parts of Europe and United States and highest (more than 25) in Netherlands, Denmark, Scotland, Ireland and New Zealand (2). From mid 1970s to mid 1980s, the mortality rate didn’t change greatly in many of the countries with high rates whereas increase occured in many of the countries with low rates resulting in narrowing of the difference in cancer incidence globally. In contrast to mortality rates which generally exist at the national level, incidence rates vary from four to five fold. Rates are lowest in parts of China, Japan and India (less than 30), they are intermediate in South America, the Carribean and Eastern Europe and highest in Western Europe, Canada and North America (3). Geographic variation is apparent within many countries. Rates in urban areas generally exceed those in neighboring rural areas (4). The risk of breast cancer among migrants approaches that of the native born population and is affected by the time interval since migration. The risk is further modified among subsequent generations (5). The risk of breast cancer increases rapidly with age during the childhood bearing years. After menopause the rates continue to increase but at a less rapid pace. Breast cancer incidence is highest among whites as compared to blacks. Rates among Asian Hispanics are about half those of whites (4). Recent figures show that breast cancer has become increasingly common in Japanese women after residence for a generation in the United States.
In recent years, the survival rates among breast cancer diagnosed patients has increased due to early diagnosis at localized stage. The survival rate is 86% in patients diagnosed at localized stage and less than 20% among those suffering from malignant cancer (6).

Rapid urbanization and adaptation of westernized life style by developing countries like India has led to the rapid increase of cancer incidence in these countries. It has been estimated that the cancer incidence in developing countries may be comparable to its western counterpart after a gap of few decades. This is a cause for serious concern and leads us to investigate the potential risk factors.

3.0 RISK FACTORS

Breast cancer is generally recognized as a disease that occurs more often among women of upper social class as measured by either educational status or family income. Studies seem to indicate that these associations largely reflect the effect of correlated life style factors such as, later stages of first child birth. Unmarried women over age of 40 have been found to have high risk of breast cancer than married women; an association reduced with child bearing (7).

This disease is more common in developed countries and affluent societies which suggests that there are some factors in the life style of the developed and affluent societies which lead to breast cancer. A brief account of various risk factors has been discussed below:
3.1 Familial Factors

Breast cancer has a strong correlation with the families having a member suffering from the disease. Familial factors are responsible for early onset of cancer. The localization of the breast cancer gene on the q arm of the chromosome 17 revolutionized the research in this area (8). The impact of identification of the breast cancer gene on prevention has yet to be determined, especially because only about 5% of all breast cancer are thought to result from genetic predisposition.

3.2 Reproductive Factors

A late maternal age at first child birth is an important determination of breast cancer risk. According to MacMohn et al. women giving their first child birth after an age of 30 years were shown to have approximately twice the risk of those with first child birth before the age of 18 years (9).

3.3 Menstrual Factors

It is evident from various studies that women with an early onset of menarche are at an increased risk of breast cancer and those who begin menstruating before an age of 12 years have approximately a 50% higher risk than those with menarche at an age 15 or later (10). Women with late menopause have an increased risk of breast cancer. The relative risk is approximately twice for natural menopause before the age of 45 years (10).
3.4 Lactation

Lactation has been increasingly reported to offer protection against breast cancer development. Cumulative number of ovulatory cycles are directly related to breast cancer risk. A beneficial effect of long duration of nursing would be expected because nursing results in substantial delay in restabilization of ovulation following a completed pregnancy (11,12).

3.5 Weight

In addition to menstrual and reproductive risk factors, a strong correlation exists between weight and breast cancer risk. The relation is critically dependent on age and has little or no risk associated with increased weight, but by age 60, increase in weight results in increase in risk of breast cancer (13).

3.6 Hormonal Factors

The association of breast cancer with cyclic ovarian activity implies that estrogen is important in the pathogenesis of the disease (14). Evidence has also accumulated that the other major ovarian hormone progestrone may also be an important factor in increasing breast cancer (15).

3.7 Contraceptives

The role of contraceptives on the risk of breast cancer is not clear at this juncture (16-18).
3.8 Dietary Factors

The incidence of breast cancer varies more than five fold around the world and the offsprings of the migrants moving from a country with low breast cancer risk to a country of high incidence acquire a rate similar to those of the new country (19,20). These observations suggest that environmental and life cycle influences are important in etiology of breast cancer. The dominant hypothesis linking diet to breast cancer has been that high fat intake increases the risk (21). Other risk factors include alcohol intake although it has been controversial, but substantial evidence has been accumulated to support positive association (22,23).

4.0 BIOLOGY OF THE BREAST CANCER

An understanding of the morphology and physiology of breast and the many endocrine inter-relationships are both essential to study the pathophysiology of the breast and the management of the benign, preneoplastic and neoplastic disorders. There are two major categories of breast carcinomas 1) Fibroadenoma and 2) Adenocarcinoma.

Fibroadenoma: Fibroadenoma originates possibly from the fibroblast (24) (Fig.1). It is detected by palpable mass of nodular density. The palpable mass is classically well defined, rubbery in texture and mobile. It has a well circumscribed nodule that may or may not contain coarse calcification. Fibroadenoma is believed to represent hyperplastic process that involves single terminal ductal-tubular unit and its surrounding connective tissue (25,26). Lesions enlarge and adjacent ductal-lobular units are incorporated by the excessive over growth of the connective tissue. Occassionaly fibroadenomal stromal cells have chromosomal abnormalities, but no
such abnormality is seen in the case of epithelial unit (27). Clonal analysis of fibroadenomas have shown that, their epithelial and stromal cell components are polyclonal, suggesting a hyperplastic rather than a neoplastic process (28). The exact cause of the fibroadenoma is unknown but it is believed to be due to hormonal imbalance, particularly, the progesterone level is found to be lower in women with fibroadenoma, while estrogen levels remain unchanged (29-30).

**Adenocarcinoma**: Adenomas are benign, well circumscribed tumors composed of epithelial elements with sparse inconspicuous stroma (31) (Fig.2). The later feature differentiates these lesions from fibroadenomas, in which stroma is an integral part. Adenomas can be either tubular or lactating. Tubular adenomas occur in young women, as well defined, free movable nodules that clinically resemble fibroadenomas (31). Tubular adenomas are separated from adjacent breast tissue by pseudocapsule and are composed of proliferation of uniform, small tubular structures with scanty amount of intervening stroma. Lactating adenomas occur in one or more freely movable masses during pregnancy or in post-partum period (31). They are well circumscribed and lobulated and on cut section they appear tan and softer than tubular adenomas. These lesions have lobulated borders and are composed of glands lined by cuboidal cells with secreting activity identical to lactational changes, normally associated with breast tissue during pregnancy and perperium.

### 5.0 Models Employed for Breast Cancer Study

#### 5.1 *In vivo* models

Many aspects of experimental breast cancer research require the use of appropriate animal models since reproducing the complexity of the endocrinologic
environment of the pituitary-adrenal-ovarian axis is beyond the scope of the current
in vitro technologies.

Most experimental animal models of breast cancer research are limited to rodents. Several different groups of rodent models are available for experimental breast cancer research. These include chemically induced [e.g. 7,12-dimethylbenz(a)anthracene (DMBA), N-nitrosomethylurea (NMU)] rat mammary carcinoma, virally induced mammary tumors, human tumor xenografts and transgenic mouse model. A choice of appropriate model and realistic assessment of its limitation are critical for its adequate and appropriate experimental design. As Siemann quotes "Choose the model to address the question rather than force the question on the tumor model" (32).

5.1.1 7-12-dimethylbenz(a)anthracene (DMBA) induced rat mammary tumorigenesis

DMBA is a potent inducer of mammary carcinomas (Fig.3). DMBA is generally administered by oral route, frequently as a solution in peanut oil; 20 mg per animal produces a final incidence of 100% adenocarcinomas generally within 10 to 15 weeks (33). The mammary tumor arises in the epithelium of the terminal end buds which are comparable in structure to the terminal ductal-lobular unit in the human breast (34). The tumors are generally ductal carcinomas and intraductal papillomas. DMBA is highly lipophilic and requires metabolic activation for its carcinogenicity. Co-administration of agents that alter its hepatic activation can influence subsequent tumor incidence. These apparent effects on tumorogenicity may be considered
artifactual since pharmacologic effects are specific to the carcinogen used. The potential for such artifacts requires careful experimental design while using DMBA, in studies with agents that could alter hepatic function or dietary studies using high fats (34).

5.1.2 N-nitrosomethylurea (NMU) induced mammary tumorigenesis

The ability of NMU to produce mammary tumor was reported by Gullino and colleagues about 20 years ago (35). NMU induces mammary carcinomas in rodents when it is administered subcutaneously or intravenously at 50 mg/kg (Fig.4). Tumor incidence and latency periods are comparable to that of DMBA administration and also exhibit, steroid hormones and prolactin responsiveness (36). As NMU does not require metabolic activation, there are fewer concerns regarding co-administration artifacts compared to DMBA. About 75% of rodent mammary tumors induced by NMU exhibit altered ras expression and its activation (37). However this occurs during initiation (38). The incidence of altered ras expression in human breast cancer is approximately 20% and it represents rare alleles or slight over-expression (38). Furthermore its role in human breast cancer initiation, promotion and progression remains unclear (39,40). This is in contrast with potent transforming ras mutation observed in NMU induced rodent tumors (37,41). The high incidence of ras activation potentially reduces the utility of NMU induced rodent tumors for signal transduction and mechanistic studies because of the probability that ras/G protein mediated pathways will be predominately high. The high incidence of activated ras increases the likelihood that data from such mechanistic studies could be heavily skewed; although this limits the studies regarding the ability of certain agents (e.g.
tumor promoters) to increase the incidence of \textit{ras} expression. Further, it may prove to be a good model for studying treatments that could either reduce \textit{ras} expression or utilize \textit{ras} mediated signal transduction pathways.

5.2 \textit{In vitro} models

Breast cancer cells that grow \textit{in vitro} represent one of the most widely used experimental models of breast cancer. For many studies, these models provide the only means to address a specific hypothesis. Most of the breast cancer cell lines can be easily maintained and studied \textit{in vitro} and are generally stable with respect to their endocrine responsiveness \textit{in vitro} and \textit{in vivo}. Our current understanding of the way in which breast cancer cells respond to estrogenic stimuli is the direct result of \textit{in vitro} studies with human breast cancer cell lines. Breast cancer cell lines are generally considered in terms of their estrogen receptor (ER) content, i.e., ER - positive (ER\textsuperscript{+}) or ER - negative (ER\textsuperscript{-}). This largely reflects the clinical value of steroid hormones expression in predicting the response to endocrine therapy. In addition however, other characteristics of the human breast tumors that tend to follow ER status frequently exhibit a similar pattern to cells growing \textit{in vitro} and \textit{in vivo} (42).

5.2.1 Steroid dependent (ER\textsuperscript{+} / ER\textsuperscript{-}) breast cancer cell lines

Approximately 30\% of all breast cancer patients respond to endocrine manipulation. The overall response rate to anti-estrogens increases to 70\% or more in patients whose tumors express receptors for both estrogens and progesterone (43-45). To define the mechanism of endocrine therapies, and to develop and screen
new agents and therapies require models that exhibit an endocrine response profile comparable to that found in breast cancer patients. In this regard, steroid dependent breast cancer cell lines have been most useful in studying the growth regulatory effects of estrogens, antiestrogens, progestins and antiprogestins. These cell lines are characterized by dependence on estrogens for growth \textit{in vitro} and \textit{in vivo} and sensitivity to the growth inhibitory effects of antiestrogen and progestational drugs. In general, steroid dependent cell lines are poorly invasive and non-metastatic in athymic nude mice.

\textbf{5.2.1.1 MCF-7 cell line}

The MCF-7 cell line is most widely used and best characterized of human breast adenocarcinoma cell lines. The mitogenic effects of $E_2$ in human breast cancer cells \textit{in vitro} were initially defined as well as inhibitory effects of antiestrogens were studied (46,47). MCF-7 cells growth is inhibited by luteinizing-hormone releasing hormone (LHRH) analogs (48) and retinoids (49-51). The widely reported $E_2$ dependence for exponential growth both \textit{in vitro} and \textit{in vivo} has provided this cell line with a central role in the study of endocrine responsiveness and malignant progression \textit{in vitro}. This model has contributed much to our current understanding regarding the mechanism of action of estrogen and anti-estrogens and their role in regulating the proliferation of hormone dependent breast cancer cells. MCF-7 cells were established from plural effusion (52) arising in a post-menopausal women with breast cancer. The patient had received both radiotherapy and endocrine therapy before appearance of effusion. In addition to expression of ER (52,53), MCF-7 cells also express $E_2$ inducible progestrone receptor (PR) (53,54) as well as cellular receptors for androgens (53) LHRH (48,55), glucocorticoids (53), insulin (56), retinoic acid receptor
(RAR-α and RAR-γ) (57) and prolactins (58). The expression and secretion of several growth factors and their receptors have also been described in detail including insulin-like growth factors (IGFs) (59-62), the type I and type II IGF receptors (63,64) and several IGF-binding proteins (65-67), transforming growth factors-α (IGF-α) and epidermal growth factor receptors (EGFR) (68-70), several fibroblast growth factors (FGF) (71), FGF receptors (72), platelet derived growth factors (PDGFs) but not PDGF receptors (73). The expression of many of these growth factors or their respective receptors is strongly E₂ regulated in MCF-7 cells (74). The expression, secretion and regulation of this wide variety of receptors and ligands have made MCF-7 cells a valuable model for the study of the role of growth factor and growth factor receptor expression in the proliferation of breast cancer cells. E₂ dependence for growth, antiestrogen sensitivity and low metastatic potential of MCF-7 cells have led to the hypothesis that they represent an early breast cancer phenotype (75,76). MCF-7 cells are excellent models to study the process of malignant expression because they can be subjected to appropriate endocrinologic and physiologic selective pressures for the derivation of the variants with more progressed phenotypes (Fig.5).

5.2.2 Steroid-Independent breast cancer cell lines

The estrogenic requirements of the MCF-7, T-47D and ZR-75-1 cells for growth in vitro and in vivo may not adequately reflect the endocrine environment of many breast tumors in post-menopausal women. Several breast cancer cell lines and variants of MCF-7, ZR-47-1 and T-47D cell lines have been generated, that no longer require estrogenic supplementation for growth. These continue to express estrogen
receptor (ER) and progesterone receptor (PR) and also retain responsiveness to endocrine agents. Several steroid independent and steroid responsive cell lines and their variants exhibit properties that more closely resemble breast tumors in patients than those of the steroid dependent cell lines.

The malignant steroid responsive cell lines have been established, not from solid tumors but from malignant effusion. Although such effusion occurs with 26% to 49% frequency in breast cancer patients (77-78), they may not be fully representative of all solid tumors. Despite the likely metastatic origin of these cells, the ER$^+$ cell lines from these sites are rarely metastatic in vivo, even in severely immuno-compromised animals.

These in vitro models in the form of human breast cancer cell lines and as human xenografts play a central role in most basic and pre-clinical breast cancer research. They have been widely used to investigate the cellular and molecular events associated with endocrine responsiveness, malignant progression, invasiveness and metastatic potential. With the increased restriction imposed on the use of vertebrate animals and relatively limited number of species that develop spontaneous mammary carcinomas, it seems likely that emphasis on the in-vitro use of human breast cancer cell line will increase in the coming decades.

5.3 Transgenic mouse model

Transgenic mice provide a powerful means to investigate the function of over-expression of a gene in-vivo. Basically, the gene is inserted into fertilized embryo which is then transplanted into appropriate pseudo pregnant, foster mice.
Reproductively viable homozygotes or heterozygotes can be mated to maintain the transgenic stock. This approach has several advantages and disadvantages. For secreted factors (e.g. growth factors) it may become difficult to distinguish among endocrine, paracrine and autocrine effects. The level of expression produced in transgenic mice can greatly exceed the observed level in normal and malignant tissues, since all cells potentially express the gene. The tumors can be multifocal or polyclonal. Polyclonality is rare in human breast tumors (76). Expression can also depend on the promoter construct used with various tissues expressing different levels of transgene. Different mice strains, may also account for some of the apparently conflicting observations among groups generating transgenic mice with comparable transgene construct. It was attempted to use tissue specific promoter to restrict over-expression in appropriate target tissue. For mammary glands this has generally involved use of mouse mammary tumor virus-long terminal repeat (MMTV LTR) (which is not totally specific for mammary gland) (79).

6.0 THERAPY

Breast cancer is a heterogeneous disease that has a propensity for systemic involvement and commonly has a long natural history. Due to this long natural history and its onset typically at middle age, it is difficult to demonstrate in a strict sense that, breast cancer is a curable disease. It is clear, however, that considerable percentage of patients with treated breast cancer (particularly when detected at early stage) live their lives without further evidence of the disease. Despite improved techniques for breast cancer screening, many women continue to be diagnosed at a stage when metastasis has already occurred, either because of lack of diagnostic facilities or because of early spread of small tumors. Treatment of breast cancer
involves a collaborative effort between surgeons, radiologist, pathologists and medical oncologists.

6.1 Surgical Treatments

Radical mastectomy an en-bloc resection of the breast, the overlying skin the pectoral muscles and the entirety of the axillary contents, had two major advantages. It was technically possible in most women with breast cancer, and even at advanced stage, it was an effective means of obtaining local control of primary tumors. Modified radical mastectomy is most common operative treatment for patients with invasive breast cancer. The term, modified radical mastectomy, is used to describe variety of surgical procedures, but all involve complete removal of the breast.

6.2 Radiation Therapy (RT)

The use of radiation therapy (RT) for breast cancer began at nearly the same time as surgical treatment. Possible complications of radiation therapy include arm edema, bronchial plexopathy, decreased arm mobility, soft tissue necrosis, rib fracture, radiation pneumonitis, carcinogenesis and radiation related heart disease. Breast conserving treatment is to remove the bulk of tumor surgically and to use moderate dose of radiation to eradicate any residual cancer.

6.3 Tamoxifen

Tamoxifen is an antiestrogen that binds to the estrogen receptor resulting in the altered RNA transcription, decreased cell proliferation and partial estrogen agonist
activity. It also causes mouse mammary cell in culture to become apoptotic rather than secretory and causes basement membrane alteration (80). Additional mechanism by which tamoxifen prevents the development of new primary breast cancer are complex and may include modulating the production of transforming growth factors, decreasing the circulatory level of insulin like growth factor, increasing circulatory levels of sex hormone binding globulin which may decrease the availability of free estrogen, removing stimulus for tumor cell growth and increasing the level of natural killer cells (80).

6.4 Chemotherapy or Adjuvant Therapy

Adjuvant therapy is defined as the administration of cytotoxic chemotherapy or the use of ablative or additive endocrine therapy after primary surgery of breast tumor to kill or inhibit clinically occult micro-metastases. These metastasis which are rarely evident on routine radiographs and scans at the time of diagnosis, account for high treatment failure rates in breast cancer patients treated only with local modalities such as surgery and irradiation.

Many active agents are available for the treatment of metastatic breast cancer, and agents with higher activity historically are anthrcyclins (doxorubicin), cyclophosphamide, methotrexate and 5-Fluorouracil (5-FU). In addition, the vinca alkaloids, etoposide platinum, ifosfamide and mitomycin are also active. More recently pacilitaxel (Taxol) has become widely used as an agent particularly effective in anthracycline-resitant tumors. Newer promising agents include other taxanes (such as taxolerete, the camptothecins, the anthrapyrazoles and navelbine).
6.4.1 Doxorubicin

The anthracyclines have long been considered the most active agents in the treatment of breast cancer. An anti-tumor, antibiotic, doxorubicin (adriamycin) is most widely used of these agents (Table 1). Its mode of action is to intercalate within DNA and inhibition of topoisomerase-II (81). When doxorubicin is used as single agent in untreated patients with metastatic breast cancer, response rates range from 40% to 50% (82-84). Combination regimens with doxorubicin increase the response rate. Some combination include doxorubicin with cyclophosphamide (CA); with cyclophosphamide, methotrexate and 5-FU (CAMF); with cyclophosphamide, methotrexate, 5-FU and prednisone (CAMFp); with cyclophosphamide and vincristine (CAV); with cyclophosphamide and 5-FU (CAF) and vincristine added to CAMFp (CAMFVp) (85-88).

6.4.2 Taxanes

Paclitaxel (taxol) is a novel chemotherapeutic agent derived from the western yew tree *Taxus brevifolia* and most widely used taxane at present (Table 1). It has unique mechanism of action and promotes microtubule assembly, in contrast to vinca alkaloids which prevent microtubule assembly (89). Paclitaxel also shifts equilibrium to stabilize the polymerized assembled form and thus, prevents cell division (89). The taxane ring has been isolated from the natural products and has been synthesized in the laboratory. Paclitaxel was initially discovered in 1960 in the National Cancer Institute USA (NCI) screening project but was abandoned because of its toxicity and solubility problems (89). New advances have overcome those problems, although this
drug is still associated with significant side effects like hypersensitivity along with hypotension, dyspnea, bronchospasm and urticaria (89). Neuropathy is also seen which is not unexpected for an agent that affects microtubules.

6.5 Chemohormonal Therapy

Most breast cancers are heterogeneous by microscopic, biochemical or molecular characterization. When hormonal receptor assays are performed by immuno-histochemistry, it is typical to see a certain percentage of cells that express receptors but rare to observe uniform and complete staining. It is therefore likely that tumor consists of a mosaic of hormone-dependent and hormone-independent cells. These observations led to a series of trials for examining the value of combined chemotherapy and hormone therapy in an attempt to increase cure rates.

There is an ever increasing need to look for alternative targets for chemotherapy. One such target which is being extensively employed for breast cancer treatment is the polyamine biosynthetic pathway. This pathway remains an attractive target for anticancer therapy. The existing literature is related to this pathway as a potential target for chemotherapy of breast cancer is reported below.

7.0 POLYAMINE BIOSYNTHETIC PATHWAY

Polyamines are low molecular weight polycations essential for growth and differentiation of all living cells (90-96). Generally polyamines include putrescine, spermidine and spermine. The levels of polyamines are generally found to be high in
tumors than in normal tissue (93), thus making polyamine biosynthetic pathway an attractive target for anticancer therapy (97,98).

7.1 Polyamine Biosynthesis

The polyamine biosynthetic pathway in living animal systems including animals, plants and bacteria has been extensively investigated and reviewed (96-101). The mammalian pathway for polyamine biosynthesis starts from ornithine which is formed by the action of arginase on arginine. Decarboxylation of ornithine by ornithine decarboxylase (ODC) produces putrescine. Putrescine is converted into spermidine by the action of an amino-propyl-transferase called spermidine synthase. A second amino-propyl-transferase, termed as spermine synthase, adds an additional amino-propyl moiety to spermidine forming spermine. The source of these propylamine groups is decarboxylated S-adenosyl methionine (Adomet) which is produced by S-adenosylmethionine decarboxylase (AdometDC). The other product of amino-propyl-transferase reaction is 5'-methylthioadenosine (MTA). MTA is broken down by the action of MTA phosphorylase and resulting adenine is salvaged and returned to purine nucleoside pool. The amino-propyl-transferase reactions which form spermidine and spermine are effectively irreversible but these compounds can be converted back to putrescine by combined actions of two enzymes spermidine/spermine N-acetyltransferase and polyamine oxidase. Polyamines can be degraded by different amine oxidase via so called terminal catabolism, which means that the product can be converted back to their precursors (Fig.6).
7.2 Enzymes of the polyamine biosynthetic pathway

7.2.1 Ornithine decarboxylase

Ornithine decarboxylase (ODC) EC 4.1.1.17 is the first and the rate limiting enzyme in the polyamine biosynthetic pathway and catalyzes the conversion of L-ornithine to putrescine. ODC activity was first discovered in the extracts of *Escherichia coli* (102). It was also found in plants (103) and protozoans and variety of mammalian tissues (104). In eukaryotes, ODC is a cytosolic enzyme. The basal level of ODC activity in resting cells is very low. Upon growth stimulation ODC activity increases several hundred fold. Yet ODC remains a minor cellular component usually representing less than 0.0001% of total cellular protein. In androgen stimulated mouse kidney which contain the highest amount of ODC of all mammalian tissue, ODC may represent 0.05% of soluble protein after maximal stimulation. The complete amino acid sequence of mouse, rat and human ODC has been deduced from the nucleotides sequence of genomic DNA and cDNA (105). The encoded protein contains 461 amino acid residues having molecular mass of 51 kDa and that represents the subunit of homodimeric native enzyme. Each sequence has 12 cysteine residues, consistent with the requirement of high thiol concentration for ODC activity. The overall homology of amino acid sequence of rat, mouse and human ODC protein is greater than 90%. However, the location of the catalytic site in ODC has not been established conclusively, but most of the hydrophilic part of the enzyme comprising amino acid residue 290-340, appears to be important for the catalytic activity. A range of pH optima from 7.0 to 8.1 has been reported for rat liver ODC depending upon purity of the enzymes (106) or the presence of thiol in the medium (107). Mammalian ODC uses L-ornithine (and L-lysine with much higher Km values) as
substrate and also requires pyridoxal phosphate (PLP) as a co-factor. A thiol reducing agent like dithiothreitol is needed for maximal activity (108).

Ornithine decarboxylase is considered to be a key regulatory enzyme of the polyamine biosynthetic pathway because its activity is highly regulated in the cells and it responds to wide variety of stimuli (109). The rapid and profound increase in ODC activity in response to hormones, peptide growth factors, regenerative stimuli and other drugs was due to an increase in ODC protein (108). A part of the increase may be due to stabilization of the protein but most of it is due to elevated rate of synthesis. In some cases this has been correlated with greater content of mRNA (110). However it is not clear to what extent the greater mRNA content reflects the changes in the transcription, processing or degradation of the mRNA or whether the increase is entirely sufficient to account for enhanced synthesis of ODC protein. ODC levels are strikingly repressed by polyamines. It has been demonstrated that decay of ODC proteins is caused by polyamines due to both suppression of its synthesis and acceleration of its degradation (111). Changes in ODC are not accompanied by the change in the content of mRNA and appear to be due to alteration in its rate of translation and in the turn over of the protein. The rapid turn over rate of ODC which may be as short as five minutes, can be explained by the presence of two PEST regions in the amino acid sequence. The PEST sequences are characteristic of many protein exhibiting short intracellular half life (112). The two PEST sequence found near C-terminal region may account for the intracellular instability of the enzyme.

A novel type of negative control of ODC is one or more inhibitor proteins discovered by Heller and Canellakis (1981) and named antizyme by them (113). This
protein binds with ODC with very high affinity forming a complex that can be observed during ODC deregulation and co-relates directly with the half life of the enzyme. The amount of the antizyme is increased in response to exposure to putrescine and other diamines (113). However, it appears that spermidine and spermine are actually more important physiological regulators of ODC activity than putrescine itself (114,115). A number of other mechanisms for regulation of ODC activity have been proposed including a variety of post-transcriptional modification (99) but none of these have been shown unequivocally to occur in vivo. ODC can be phosphorylated at serine residue 303 but this phosphorylation has not yet been shown to lead to any significant change in the enzyme activity or stability (116,117).

7.2.2 S-adenosylmethionine decarboxylase

S-adenosylmethionine decarboxylase (AdometDC) EC 41.1.50 decarboxylates S-adenosyl methionine from which the amino-propyl moiety is derived to form spermidine from putrescine and spermine from spermidine by respective amino propyl transferase. In contrast to ODC, AdometDC is not dependent on pyridoxal phosphate as a co-factor, but it relies on covalent bound pyruvate as prosthetic group (118). The pyruvate moiety of AdometDC is synthesized as an inactive 38kD proenzyme containing 333-334 amino acid residues which is then cleaved at unknown site to yield two polypeptides of 32 kD and 6 kD (119). The cleavage presumably generates pyruvate from an internal serine residue. The pyruvate is apparently localized at the N-terminus of the large subunit. Purified preparation of mammalian AdometDC have only revealed the 32 kD subunit. However, it was demonstrated recently that the smaller peptide is also a subunit of active enzyme. This subunit is precipitated with
the antibody raised against the pure enzymes and contains a sequence identical to
sequence found in pure bovine AdometDC (120). AdometDC activity is induced in
response to a variety of stimuli including hormones and mitogens (96,99). The
induced activity of AdometDC is the result of increased accumulation of enzyme
protein (121). There is increase in mRNA content during stimuli induction but this
is not sufficient to account for greater rate of synthesis, indicating that translation
regulation may play an important role in this control. The increase in translational
efficiency seems to arise from selective increase in the rate of initiation, leading to
more ribosomes per polysomes encoding AdometDC mRNA (122). The nature of the
enzyme appears to be hetero-tetrameric, consisting of two small and two large subu-
nits. AdometDC, like ODC, also turns over quite rapidly, due to the presence of a
strong PEST region at residue 243-269 within the large subunit. AdometDC is highly
regulated by putrescine, spermidine and spermine but here putrescine acts in a
different manner than that of other polyamines. Putrescine induces AdometDC
directly (96,118) and it also increases the conversion of 38 kDa proenzyme to 32 kDa
enzyme subunit (96,115). In this way, rise in putrescine levels results in the
production of decarboxylated adenosyl-methionine, which can be used as substrate for
spermidine synthase to convert putrescine into spermidine.

7.2.3 Spermidine synthase and Spermine synthase

The aminopropyl-transferases, which transfer amino-propyl moiety to
putrescine form spermidine and then another one to spermidine to form spermine by
respective aminopropyl transferases known as spermidine and spermine synthases
respectively, have been fully characterized from bovine brain and from rat
prostrate and liver (123). Spermidine synthase consists of two subunits of Mr about
36000 and spermine synthase has two subunits of Mr 44000. Detailed kinetic studies of both the spermine synthase and spermidine synthase reaction have now been carried out using homogeneous preparation of these enzymes (123,124). These have confirmed earlier reports that these enzymes have Km value for decarboxylated AdoMet of 0.1-1.1 μM and that it is likely that they are regulated by the availability of this substrate and their competition for it. The product methyl transferase acetate (MTA) that inhibits its activity is well established (125) and rate of spermine synthesis is likely to be affected by accumulation of MTA in these cells which MTA phosphorylases (126,127).

### 7.2.4 Acetylation and Interconversion of Polyamines

Polyamine acetylation can be catalyzed by spermidine/spermine-N-acetyltransferase (SAT) along with nuclear histones acetylase (128). These enzymes are quite different and have been differentiated by their substrate specificity (129) by the use of specific antibodies (130) and their differential response to inhibitors (136). SAT has been studied in detail in terms of its specificity, kinetics and active site using homogeneous preparation (134). The acetylation of polyamines via SAT appears to be limiting factors in the degradation and interconversion of polyamines. N¹-acetylsperrmidine and N¹-acetylsperrmine are rapidly degraded by polyamine oxidase (FAD-dependent). Although this oxidase will act on non-acetylated polyamines under certain artificial conditions in vitro, it is clear that the acetylated, derivatives are true physiological substrates (132,133). SAT activity can be induced by wide variety of toxic stimuli (129). These inductions are brought about by an increase in the amount of enzyme protein which occurs because of both a large
increase in the rate of synthesis and in some cases by decline in the rate of
degradation (129,133). SAT also has short half life as ODC (129) and is regulated by
phosphorylation/dephosphorylation mechanism (135).

8.0 INHIBITORS OF POLYAMINE BIOSYNTHESIS AND THEIR USE IN CANCER
CHEMOTHERAPY

Polyamine biosynthesis can be inhibited by a number of inhibitors of its
enzymes and by analogs of polyamines which are discussed here (Table 1).

8.1 Inhibitors of Ornithine Decarboxylase

Many substances which are potent ODC inhibitors are developed and described
but the only compounds that have been demonstrated unequivocally to have useful
pharmacological and experimental potential are the enzyme activated irreversible
inactivators of which difluoromethylornithine (DFMO) is a prototype (136). Other
potent inhibitors have also been made based on the synthesis of aminooxy-derivatives
of putrescine and shown to be effective in culture cells (137-139). The selection of
ODC as a major target for chemical intervention was based upon several
experimentally verified facts: i) The activity of ODC under most circumstances,
though admittedly not always, is the lowest among enzymes engaged with
synthesis of polyamines. ii) Putrescine, the product of reaction, is required for
activation of AdometDC (140). iii) Putrescine is needed for processing of the
AdometDC peptide to active enzyme (141,142). Metcalf and his co-workers
succeeded in synthesizing α-DFMO or eflornithine, a derivative of amino
acid ornithine (143). It belongs to the class of the so-called mechanism based
inhibitors or suicidal inhibitors. As implied by the mechanism of action, \( \alpha \)-DFMO serves as a real substrate for ODC and in fact, is decarboxylated during the reaction. As a result of decarboxylation, however, a series of electron rearrangements occur leading to an irreversible inactivation of ODC owing alkylation of its active center. Based upon the mechanism of action, \( \alpha \)-DFMO is entirely specific to ODC and doesn't interfere with any other enzymatic reaction, using ornithine as a substrate. A lot of work has been done describing the anti-proliferative nature of this compound in animal cell lines. The inhibition of ODC by \( \alpha \)-DFMO in rapidly growing cells resulted in depletion of putrescine and spermidine while the concentration of spermine rose a little (99,144).

8.1.1 DFMO as an antitumor and chemopreventative agent

DFMO was found to be very effective in some animal tumor models but when used as a single agent or in combination with other agents, including interferon in phase I and phase II trials, it showed little activity in most of the cases (98,100,136,145). However, an exception was demonstrated in patients with recurrent glial tumors, where treatment of DFMO alone or combined with bis(chloroethyl)nitrosourea (BCNU) or methylglyoxal bis(guanyl) hydrazone (MGBG) produced a significant response (146-148). There are several possible reasons for the lack of response to DFMO in these clinical trials. A major factor may be availability of polyamines from other sources. Polyamines can be obtained from the diet from products of intestinal microbial flora and via polyamine oxidase (PAO) spermidine/spermine N\(^1\)-acetyltransferase (SSAT) of retroconversion pathway from other cells in the body having significant spermine stores (149-151). Much stronger
antitumor effects of DFMO have been obtained in animal models when the drug is combined with regimes designed to minimize the availability of polyamines from these sources. These regimes include the use of polyamine deficient chow, antibiotics to reduce the contribution of polyamines from intestinal micro-organism and PAO-inhibitor to prevent recycling (150-152). The extent to which such procedures can be duplicated with human cancer patients and degree of the therapeutic effects that might result remains to be determined. DFMO administered well after initiating agents, greatly reduces tumor development in rodent treated with wide variety of chemical carcinogens and tumor promoters. The production of tumor of skin bladder, stomach, intestine, colon, oral cavity and mammary glands has been blocked by DFMO treatment in animal models (153). ODC activity is enhanced by variety of tumor promoters and ODC may act like an oncogene if its expression can be elevated to high enough levels with carcinogens treatment. Several other chemopreventive agents such as retinoids and piroxicam are also known to reduce ODC via indirect mechanism affecting the level of ODC protein. These results provide a strong rationale for the possible use of DFMO as a chemopreventive agent in population at high risk for the development of neoplasm (136,153,154).

8.2 Inhibitors of S-adenosylmethionine decarboxylase

Although AdometDC has received less attention than ODC as therapeutic target since DFMO became widely available, there has been long history of interest in inhibitors of AdometDC ever since it was discovered more than two decades ago. MGBG, a known antitumor agent, is a powerful competitive inhibitor (155,156) which in fact is the first inhibitor of any of the polyamine biosynthesizing
enzymes to be discovered. MGBG [methylglyoxal bis(guanyl)hydrazone] still remains probably as the most important model compound for design of an inhibitor for AdometDC. Although at first sight MGBG does not resemble the nucleoside-S-adenosylmethionine, this compound nevertheless acts as a competitive inhibitor of AdometDC (157). Unlike DFMO which is remarkably specific to ODC, MGBG exerts several effects that in all likelihood have nothing to do with inhibition of AdometDC or a fall in spermidine and spermine levels. This includes inhibition of diamine oxidase (94,158) and profound antimicrobial action seen at morphological as well as functional levels (159). The drug likewise affects lipid metabolism by inhibiting the carnitine-dependent oxidation of long chain fatty acids (160). Irrespective of its mode of action, MGBG exerts clear-cut antiproliferative effects on most animal cells at micromolar (1 μM to 10 μM) concentration. The compound enters the cell by using the putative polyamine carrier (161) and consequently depletes polyamines. This activates the carrier function and also enhances cellular uptake of MGBG (162). The unwarranted effects exerted by MGBG started a search which is still in progress for more suitable inhibitors of AdometDC but which abolishes inhibition of diamine oxidase. One possible way is by alkylating the molecule which appears to have affinity for AdometDC but reduction in the activity against diamine oxidase (163). Regenass and colleagues (164-167) used traditional approach of making a large number of compounds related to MGBG and then screening these compounds for those with good activity against AdometDC and less activity towards both diamine oxidase and the ability to cause mitochondrial damage, well known site for MGBG action. These compounds which are apparently specific to AdometDC including (2,2'-bipyridine)-6,6'-diacarboximidamide (CGP39937) (164) and 4-amidinoindan-1-one 2'amidino hydrazone (CGP48664) (166), are powerful inhibitors of AdometDC
and cause a major depletion of spermidine and spermine. Binding of these compounds to AdometDC leads to stabilization of enzyme protein (like MGBG) and also leads to an increase in total protein. However, inhibitors potency is sufficiently so great that AdometDC activity remains reduced even in the face of this increase (164). This contrasts with MGBG itself which produces only a modest decrease in AdometDC concentration in treated cells owing to increased content of AdometDC. CGP48664 differs from MGBG that it does not rely on polyamine transport system for its uptake and does not have strong anti-mitochondrial activity. The resistance to CGP48664 leads to AdometDC gene amplification (165) suggesting that AdometDC is indeed a major target of this drug.

8.3 Polyamine Analogs

Various polyamine analogs containing various N-alkyl substitutes have been synthesized. These polyamine analogs are found to be antitumoric in nature but then the mechanism suggested is that since they are analogs of polyamines they hamper the normal transportation of polyamines process and also compete with the binding site of polyamine at DNA or other cellular molecules, thus, disturbing natural function of polyamines which ultimately leads to cell kill.

One of these compounds is bis(ethyl)analog in which ethyl groups are present on the terminal nitrogen atoms. It was found that N\textsuperscript{1},N\textsuperscript{8}-bis(ethyl)spermidine reduced cellular ODC levels and decreased interacellular content of putrescine, spermidine, and spermine. The corresponding spermine analog N\textsuperscript{1}N\textsuperscript{12}-bis(ethyl)spermine was more active in repressing ODC and also reduced AdometDC content and brought about
almost complete loss of all cellular polyamines including putrescine. It also accumulates at concentration up to 5 fold that of spermine in control cells (168). So, it is suggested that not only polyamine deficiency but also accumulation of N¹, N¹²-bis(ethyl)spermine may be involved in the inhibition of cell growth. These effects are presumably due to analogs being recognized as polyamine by the physiological system which regulates ODC and AdometDC levels. These compounds do not support the cell growth in L1210 cells, therefore acting as inhibitor of cell growth in L1210 cells and number of other cell lines. They are particularly active as inhibitor of growth of human undifferentiated large lung carcinoma cells and N¹N¹²-bis(ethyl)spermine appears to be particularly promising for chemotherapy of such tumors. N¹,N¹²-bis(ethyl)spermine was also found to act on depleting mitochondria DNA. The other function of polyamines is to interact with DNA, so polyamine-DNA interaction can also play a major role in the development of therapeutically active polyamine analogs. Since other anionic sites in the cell bind polyamines and it is not certain that DNA is the sole primary binding site for cytotoxic effect. The characteristic that seems to relate best in this "semi-rational" approach to drug development are affinity for DNA and diminished ability to aggregate DNA versus the present polyamines. The most notable compounds developed are petamine analogs BE3333 and BE4444. These compounds have been studied extensively in human brain tumor cell line in cell culture (169) and in nude mice xenografts both for human brain tumors (170). These bis(ethyl)pentamines are stronger inhibitors of cell growth than the bis(ethyl)tetramines in vitro system (171). BE3333 is the least toxic bis(ethyl)pentamine against various human tumor xenotransplanted in to nude mice and is found to have stronger antitumor activity. Interestingly, pentamines were first identified in the thermophile Thermus thermophiles grown at high temperature (172).
while the parent compound is not naturally occurring pentamine, but studies of pentamines interactions with DNA (173) were important to the development of BE4444.

9.0 ROLE OF POLYAMINE BIOSYNTHETIC PATHWAY IN BREAST CANCER

Polyamines are reported to be essential mediators of estrogen stimulated breast cancer growth. Polyamines interfere at multiple levels of estrogen action including the association kinetics of the estrogen receptor to specific DNA sequences (174), estradiol regulated cell-cycle specific gene transcription (175) and the synthesis and/or action of estradiol modulated growth factors (176,177). Constitutive activation of polyamine pathway may provide a growth advantage to breast cancer cells, possibly by passing the need for estrogen stimulation. Polyamines may be at least one element involved in the transformation of hormone-dependent breast cancer cells to hormone-independent ones. Polyamines may have a role in more aggressive phenotype of breast cancer cells most likely by concert with activation of other oncogenic signals.

The above conclusions were substantiated by the following observations: treatment with DFMO, an ODC inhibitor, leads to regression of tumor in-vivo and also inhibits the colony of the nitrosomethylurea (NMU) induced rat mammary tumor cells cultured in soft agar (178). DFMO also inhibits estradiol, progestrone, prolactin, and growth hormone stimulated growth of tumors in soft agar (179-181). But growth inhibitory effect was completely reversed by exogenous polyamines (189-191) which reflects that polyamines also play an important role in mediating hormonal effects. Antitumor effect of tamoxifen, an antiestrogen, was reversed by exogenous
administration of polyamines (182). *In-vivo* antitumor effects of tamoxifen could not be reversed in ovariectomised mice by putrescine (183), which further reflects the involvement of polyamines in hormonal action. Polyamines inhibition by DFMO inhibited the proliferative effect of exogenously added IGF-I (176) and TGF-α (184). In a recent study, it was found that over-expression of ODC is involved in the acquisition of hormone-independent less differentiated phenotype in breast cancer (185).

Overall polyamines were found to play an important role in breast cancer growth and appear to be critical mediators of proliferative effects on mammary tumor cells induced by hormones and growth factors. There appears to be selectivity of polyamine involvement in hormone action since these compounds do not appear to be required for estrogen action, stimulation of progestrone-receptor synthesis and growth of some natural tissues such as uterus (186) and prostate (187).

This selectivity along with anti-polyamine therapy, could be exploited as a therapeutic potential. Inhibition of polyamine biosynthesis with DFMO has been shown to be quite effective in inhibiting the growth of established mammary tumors in rats as well as inhibiting promotion of tumorigenesis. Antipolyamine therapy could be employed in combination with standard endocrine treatment supported by study in which DFMO in ovariectomized mice suppressed the tumor cell labeling indices. Thus, polyamine biosynthetic pathway could prove as an effective target for chemotherapy in breast cancer.
10.0 **Antioxidants**

Antioxidants are concerned with the removal of free radical formation and thus prevent auto-oxidation of fats and oils along with the decomposition of peroxidase or inactivation of metals. There are so many different types of antioxidants present in the environment, both synthetic and naturally occurring. They are present in food stuff as food additives or in soap, cosmetics, rubber, oils product or plastics and many other naturally occurring antioxidants are present in environment or in plants e.g. selenium or retinoids etc. Generally, antioxidants are non-mutagenic and sometimes they interfere in the mutagenic process of some other mutagens. They are found to be anticarcinogenic in rats or mice when they are given prior to and/or simultaneously with certain carcinogens. There are reports in favor of the relationship between decrease in incidence of mortality from cancer of certain organs and consumption of anti-oxidants, e.g., the incidence of certain cancers in population is inversely related to the amount of selenium in the environment. There is inverse association of dietary intake of carotene or retinoids (vitamin A) and cancer incidence and there is strong inverse association between serum vitamin E and β-carotene and the risk of all histological types of cancer and squamous cell carcinoma respectively of lung. These antioxidants like retinoids and selenium have shown to have antiproliferative activity in cancer cells but still the mechanism is not clear. The antioxidants which are used in this study, selenium and retinoic acid, are discussed below.
10.1 Selenium

Selenium is an essential part of the enzyme glutathione peroxidase (GSH-Px) (Table 1). This enzyme metabolizes hydroperoxides and therefore is an essential part of the antioxidant defense system keeping the organism free of peroxide compounds mainly H₂O₂ and preventing the generation of ·OH. Since oxygen radicals such as O₂⁻ or ·OH may initiate the process of uncontrolled cell proliferation and their detoxification by increase of GSH-Px in the tissue, it may contribute to defense of organism against cancer. But Medina et al. (1983) have shown that GSH-Px plays no role in chemoprevention of mammary tumorigenesis by selenium (188). They propose that inhibitory effects of selenium may be at the level of DNA synthesis. However, Collombatto et al. (1987) have shown that selenite promotes marked increase in liver ODC activity and putrescine concentration as supposed to marked decrease in bursa, whereas spermidine acetyl transferase (SAT) activity is elevated in both tissues (189). Selenium is also found to influence other enzyme activities involved in detoxification process of carcinogenesis. Selenium deficiency increases the activity of glutathione-S- transferase (GST) in liver, kidney and duodenal mucosa of rats, however, supplementation of selenium decreases GST activity. As it is known that GST is involved in detoxification process of foreign compounds, therefore, inverse correlation between chemical carcinogenesis and this enzyme protects the selenium deficient animal against the influence of same carcinogens.

10.2 Retinoids

Retinoids are known to induce a variety of biological and biochemical effects in cells which seem to vary according to the cell type (Table 1). Amongst
these is the ability to prevent the chemical induction and growth of tumors in model system and to exert anti-proliferative effects in certain cell lines. Although the precise mechanism of retinoids action in epithelial differentiation and in cancer prevention remains to be unravelled, several hypothesis have been proposed. Retinoids exert influence on DNA synthesis, post-transcriptional glycosylation of proteins, lysosomal membrane stability agglutination and adhesive properties of transformed cells and stimulation of gap junction. Retinoids are also known to control several proteins such as ODC, plasminogen activator and alkaline phosphatase. It has also been shown that retinoic acid inhibits synthesis of ODC mRNA, possibly mediated by protein kinase C.

11.0 DRUG RESISTANCE

Drug resistance represents a major clinical problem in cancer therapy. The repeated use of chemotherapeutic agents often leads ultimately to their becoming ineffective due to onset of resistance or tolerance by target cells. This phenomena is known as drug resistance. It is of great importance from economic point of view and leads to grave consequences to public health. It also serves as a major challenge to pharmaceutical industry because development of resistance ensures that effective drugs become limited in their usefulness. The understanding of the mechanisms involved in drug resistance has been a major issue in medicine since last two decades. Despite the infinite variability in our environment, it is remarkable that drug resistance is often achieved by a relatively small number of mechanisms and it can be classified in two categories: 1) intrinsic or 2) acquired.
11.1 Intrinsic Drug Resistance

If an organism or a cell is resistant to a particular compound or drug at the time of treatment, this property is known as intrinsic resistance, also sometimes known as natural or *de-novo* resistance. This inherent or integral property of species has arisen through the process of evolution. By virtue of this characteristic feature, the organism or cell can tolerate a particular drug or environment.

11.2 Acquired Drug Resistance

Normally the cells or organism are initially sensitive to the new drug or chemical to which they are exposed. But after a prolonged exposure some of them among whole population may become resistant to the agent. The biological feature responsible for resistance is either absent from population or is not exposed in major portion of population before drug exposure. This form of resistance can arise by several different mechanisms. However, mutation and selection for a particular gene are central to this process.

12.0 BIOCHEMICAL MECHANISMS INVOLVED IN DRUG RESISTANCE

There are various mechanisms involved in drug resistance. For example, in case of changes in either drug transportation or drug detoxification, it is normally achieved by more than one mechanism and can lead to manifestation as cross drug resistance against structurally related or unrelated compound, the phenomenon is often referred to as multidrug resistance.
12.1 Decreased Drug Delivery

If the amount of drug reaching the target cell is below the cytotoxic level, the target will not be killed and hence remains unaffected by drug, e.g., in mammals the drug is delivered through blood to target tissue and the tissue like brain where the blood brain barrier is there and is therefore very difficult to target for chemotherapy. Further the vascularisation of tumor is highly variable and tumors that are poorly vascularised are difficult to target. Half life of drug in plasma is also another factor in drug delivery which is again dependent on rate of metabolism, invariably at a site separate from target cells. The metabolizing enzymes are also detoxifying enzymes and are induced by xenobiotics. Previous drug therapy or exposure to other inducing agents can exert a profound effect on drug availability.

12.2 Decreased Drug uptake

If the mechanism of drug transport is defective, the target cell will be resistant to the drug. This property can be due to lipophilicity of drug and structure of cell membrane. The drug transport inside the cell is either by passive way or through active transport. The passive uptake of drug by cell depends upon the physiological nature of membrane.

12.3 Drug efflux

If the drug is effluxed out of the cell, the cells will not be sensitive to the drug. The proteins involved in this mechanism are the main reason for the development of drug resistance to many compounds. Similarly, in human tumors the
presence of trans-membrane energy-dependent efflux pump, p-glycoprotein, can confer resistance to many anticancer drugs.

12.4 Metabolism of Drug

The intracellular concentration of drug can be reduced by metabolizing enzyme but in some cases, the drug is required to be metabolized before it exerts its chemotherapeutic effects. Thus, these enzymes can potentiate or reduce the toxicity of drug. So in some cases it is activation and in other cases it is deactivation of these enzymes that lead to drug resistance. Both the oxidation (phase I) and conjugation (phase II) enzymes play a central role in the process. The cytochrome P-450 the phase I enzyme catalyze the formation of ultimate toxic and carcinogen metabolism.

12.5 Drug Sequestration

Reduced availability of intracellular drug binding also known as drug sequestration, is one of the mechanisms of drug resistance. The increased expression of metallothione, a low molecular weight cysteine-rich protein, has been implicated in this mechanism (190).

12.6 Repair of Drug-induced Damage

Increased rates of repair of cellular damage represents an important mechanism of resistance to alkylating agents and particularly radiation. While DNA repair has been excessively studied, the replacement of protein and the repair of membranes has been essentially ignored. A number of DNA repair enzymes have been described (191) and in context of drug resistance, O^6-alkylguanine-DNA alkyl transferase has
been shown to be responsible for the resistance to methylating agents, such as N-methyl-N-nitrosourea, methylmethane sulfonate and N-nitro-N-nitrosoguanidine (192,193).

There are other mechanisms which also play an important role in drug resistance; mechanisms like increase in intracellular target site or change in structure of target so that the drug will have less affinity toward the target.

12.7 P-glycoprotein

P-glycoprotein is a 170 kD protein mostly involved in multidrug resistance phenotype and is a energy-dependent efflux protein as its expression effectively reduces the accumulation of certain anthracyclins and vinca-alkaloid. These proteins contain twelve membrane-spanning helices and two nucleotides (ATP)- binding sites. The number of processes of transmembrane segments suggest that it forms a pore-forming protein (194) and channel through which drugs can be effluxed out. The highly conservative ATP-binding site confirms it as an energy dependent transport system. P-glycoproteins are encoded by multigene family and their number varies in different species. On the basis of sequence homologies, the mdr-gene is classified in two major classes, mdr-1 and mdr-2. Involvement of p-glycoprotein in multidrug resistance is well studied and was found that it is expression of p-glycoprotein or changes that affect its function as an efficient efflux pump lead to mdr phenotype.

Thus, proper understanding of the mechanisms involved in drug resistance is important to develop an effective chemotherapeutic strategy against cancer or any disease.
13.0 AIM OF STUDY

The present work was designed to investigate the action of several chemomodulating agents on experimental mammary carcinogenesis and also to study their mechanism of action. The work is focused on the murine model system on one hand and a hormone sensitive MCF-7 human breast adenocarcinoma cell line on the other. The murine models used for this study are dimethylbenz(a)anthracene (DMBA) induced and N-nitrosomethyl urea (NMU) induced rat mammary carcinogenesis. The role of polyamine biosynthetic pathway as a target for breast cancer therapy has been worked out in detail. The influence of inhibitors of polyamine biosynthetic pathway, like difluoromethylornithine (DFMO), hormones, antiestrogens, adriamycin, different antioxidants and taxol, an inhibitor of disagggreation of microtubule assembly, has been worked out on the MCF-7 breast cancer cell line. The effects of some of these modulators on precancerous/cancerous lesion, tumor type, cell proliferation markers like ornithine decarboxylase (ODC) and polyamine levels has also been reported. The other parameters used in this study are the effects of chemomodulators on the cell cycle and DNA fragmentation leading to apoptosis. Another aspect of this study was to find out the mechanism involved in the drug resistance to difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase (ODC). This was done by raising a DFMO resistant MCF-7 cell and studying the mechanism of drug resistance in this cell line. These results will later lead to the exploration of the possible remedy against development of drug resistance to polyamine biosynthetic pathway.
Fig. 1  Fibroadenoma tissue. The tumor is well circumscribed and its separated from the adjacent best issue by a rim of dense collagen. Both glandular and stromal elements are apparent.

Fig. 2  Adenocarcinoma tissue. The tissue revealing epithelial cells entrapped in a fibrotic stroma. The cells are cytologically benign, but the pattern simulates that of invasive carcinoma.
Fig. 3  Sprague Dawley Rats with DMBA induced mammary tumor

Fig. 4  Sprague Dawley Rats with NMU induced mammary tumor
Fig. 5  A human breast adenocarcinoma cell line MCF-7
Fig. 6: Polyamine biosynthetic pathway.
Table 1  Drugs / inhibitors used in the study

<table>
<thead>
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<th>Drug / inhibitor used</th>
<th>Structure</th>
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</tr>
<tr>
<td>Taxol</td>
<td><img src="image" alt="Structure" /></td>
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<tr>
<td>DFMO Difluoromethylornithine</td>
<td><img src="image" alt="Structure" /></td>
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<tr>
<td>MGBG Methylglyxal bis(guanyl) hydrazone</td>
<td><img src="image" alt="Structure" /></td>
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<tr>
<td>CGP48664 4-aminoindanon-1-(2-amidino) hydrazone dihydrochloride monohydrate</td>
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<td>BE3333 1,15-bis(ethylamino)-4,8,12-triaza pentadecane</td>
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<tr>
<td>BE4444 1,19-bis(ethylamino)-5,10,15-triazanonaodecane</td>
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<tr>
<td>Sodium selenite</td>
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