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UMI
CHEMOPREVENTION AND THERAPY OF MAMMARY CARCINOGENESIS BY PROSTAGLANDIN H SYNTHASE-2 (COX-2) INHIBITION

DISSERTATION

Presented in Partial Fulfilment of the Requirement for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University.

By

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2001

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ABSTRACT

A promising new lead for the control of breast cancer comes from recent epidemiological studies which suggest that regular intake of nonsteroidal anti-inflammatory drugs (NSAIDs) reduces the risk of human cancers, including breast cancer. The anticarcinogenic activity of NSAIDs appears to be related predominantly to their inhibitory effects on prostaglandin H synthase (COX) isoforms (COX-1 and COX-2) related mechanisms. A growing body of evidence has shown the inducible COX-2 gene to be overexpressed in several types of cancers, including breast cancer, and suggests an association between its blockade and inhibition of tumor development and growth.

In a series of studies, the chemopreventive and therapeutic potential of the selective COX-2 inhibitor, celecoxib, was investigated in chemically (DMBA)-induced rat mammary cancer model. Celecoxib significantly inhibited mammary tumor incidence, tumor multiplicity, tumor volume and increased tumor latency, in a dose-dependent manner; and the inhibition was approximately equal during the initiation or promotion phases of mammary carcinogenesis. The chemopreventive effects of celecoxib exceeded those obtained by the nonselective NSAID, ibuprofen, or the retinoid, 4-HPR, which were used for comparison in these experiments. No sign of toxicity was observed with celecoxib use during the experimental period. Also, the administration of celecoxib to animals with established
DMBA-induced mammary tumors, caused reduction of the cancer load (CL), that is, regression of tumor volume as well as decrease in the total number of tumors. The chemotherapeutic effects of celecoxib exceeded those obtained by ibuprofen. On the other hand, the selective COX-1 inhibitor, SC560, did not cause any regression in tumor volume, and tumors continued to grow.

The results of these pre-clinical efficacy studies provide further evidence for the role of NSAIDs in chemoprevention and therapy of breast cancer, similar to what has been reported in colon cancer. The higher potency and lower toxicity of COX-2 inhibitors offer chemopreventive/chemotherapeutic advantage over the nonselective NSAIDs, and underscore their potential usefulness in the future for the prevention and therapy of human breast cancer.
DEDICATED TO MY PARENTS
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PUBLICATIONS

Research Publications


Abstracts


8. Curley RW, Jr., Alshafie GA, Abou-Issa HM, Clagett-Dame M, and Weiss KL. The development of chemopreventive stable analogs of 4-HPR metabolites and 4-HPR.


FIELDS OF STUDY

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LIST OF ABBREVIATIONS

AMF : Autocrine motility factor.
AP-1 : Activator protein-1.
APC gene : Adenomatous polyposis coli gene.
ATF : Activator of transcription factor.
BP: benzo[a]pyrene.
BPDE : benzo[a]pyrene diol epoxide.
CID : Cartilage-derived inhibitor.
C/EBP : cAMP enhancer-binding protein.
CRE : cAMP response element.
CREB : cAMP response element binding protein; cAMP regulatory binding protein.

DMBA : 7,12-dimethylbenz[a]anthracene.
ECM : Extra cellular matrix.
EGF : Epidermal growth factor.
8-OH-dG: 7,8-dihydro-8-oxodeoxyguanosine.
EPA : 5,8,11,14, 17 - eicosapentaenoic acid.
ER : Estrogen receptor.
ERet : Endoplasmic reticulum.
FABP : Fatty acids binding protein.
FGF : Fibroblast growth factor.
FSH : Follicular stimulating hormone.

HGF/SC : Hepatocyte growth factor, Scatter factor.
H$_2$O$_2$ : Hydrogen peroxide.
IGF-I, II : Insulin-like growth factor I, and II.
IL-6, 8 : Interleukin-6 or -8.
INF $\alpha$, $\beta$ : Interferon-$\alpha$, and -$\beta$.
iNOS : Inducible nitric oxide synthase.

$K_m$ : Michaelis constant : the concentration of substrate when $v_o$ is equal to one-half $V_{max}$.
$K_{cat}$ : Catalytic constant (turnover number) : the number of catalytic events per second per active site or enzyme molecule.

LH : Luteinizing hormone.

MAPK : Mitogen-activated protein kinase.
MDGF-1 : Mammary-derived growth factor-1.
MDGI : Mammary-derived growth inhibitor.
MHC-II : Major histocompatibility complex - class II.
Min mouse : Multiple intestinal neoplasia mouse.
MMTV : Murine mammary tumor virus.
MP : Metalloproteinase.
MSF : Migration stimulating factor.

NF$_k$B : Nuclear factor kappa B.

$\omega$- x : Omega minus x (or $n = x$), where $x$ is the number of carbon atoms from the methyl end of unsaturated fatty acid where a double bond is first encountered, e.g. $\omega$-3 ($n = 3$), $\omega$-6 ($n = 6$), $\omega$-9 ($n = 9$).

PDGF : Platelet-derived growth factor.
PD-EGF : Platelet-derived endothelial growth factor.
PF-4 : Platelet factor-4.
PG : Prostaglandin.
PGHS : Prostaglandin H synthase; prostaglandin G/H synthase; prostaglandin endoperoxide synthase; COX (cyclooxygenase).
PGR : Prostaglandin receptor.
PKA : Protein kinase A.
PKC : Protein kinase C.
PPAR : Peroxisome proliferator-activated receptor.
PR : Progesterone receptor.

RT-PCR : Reverse transcriptase- polymerase chain reaction.

SEM : Standard error of the mean.
SERM : Selective estrogen receptor modulator.
Sn2 : Position 2 ($\beta$) of glycerol moiety.
Sp-1 : Sequence specific (DNA-binding) protein-1.
SRC-1 : Steroid receptor co-activator-1.

TGF-\(\alpha\), -\(\beta\) : Transforming growth factor-\(\alpha\) and -\(\beta\).
TIMP : Tissue inhibitor of metalloproteinases.
TNF-α: Tumor necrosis factor-α.
TPA: 12-O-tetradecanoyl phorbol-13-acetate.

VEGF: Vascular endothelial growth factor.

\( \nu: \) Velocity or rate of a reaction \( \Delta P / \Delta t \), the relationship between the amount of reaction product (P) formed in a unit of time (t). \( \nu \) varies directly with the concentration of each substrate or catalyst. Also \( \nu = k [S] \), the rate constant of a reaction involved a substrate (S).

\( \nu_i: \) the initial velocity of enzymatic reaction measured during the initial short period of time where little product has been formed, so the reverse reaction is negligible, thereby avoid complications associated with product inhibition and slow denaturation of enzyme.

\( V_{\text{max}}: \) the maximum velocity: the value of \( \nu_i \) for a solution of enzyme (E) that is saturated with substrate (S).
CHAPTER 1

INTRODUCTION

1.1 An overview.

Several endogenous and exogenous factors appear to be involved in the development and growth of breast cancer. Identification of these factors and understanding their mechanisms of action may contribute to the prevention and treatment of this disease.

This dissertation reports the results of a series of preclinical efficacy studies that investigated, in a chemically (DMBA)-induced rat mammary cancer model, the proposal that nonsteroidal anti-inflammatory agents (NSAIDs) inhibition of prostaglandin H synthase (COX) isozymes, particularly the inducible COX-2 isoform, and consequently its prostanoid metabolic products, may significantly inhibit the process of mammary carcinogenesis.

In the first chapter, the epidemiology of human breast cancer is reviewed, followed by discussion of the natural history of mammary carcinogenesis. Next, the COX isozymes and the prostanoid cascade are discussed, followed by general discussion of the concept of cancer chemoprevention, finally, NSAIDs are discussed in general.

The second and third chapters cover the chemopreventive and chemotherapeutic experimental designs used, and the results obtained. In the final chapter, the significance of
these results in relation to the proposed roles of COXs in carcinogenesis and NSAID antineoplastic effects is discussed.

1.2 Epidemiology of Breast Cancer.

1.2.1. Incidence and Mortality.

Breast cancer is the leading female malignancy in the United States, and despite intensive effort in cancer control, it remains the second leading cause of cancer deaths among American women (Singletary et al., 1998; Cancer Facts, 2000). The reported lifetime cumulative risk of developing breast cancer is 12.6% (that is, one in eight women) by age of 95, and the risk of dying of the disease is about 3.6% (Beers et al., 1999). After an annual increase of about 4% in the 1980s, the female breast cancer incidence rates have leveled off in the 1990s to about 111 cases per 100,000 (Ries et al., 1999). Some 182,000 new cases of invasive breast cancer in women were estimated to occur in the U.S. during 2000, and the diagnoses of about 1,400 new cases of breast cancer in men were estimated in that year (Cancer Facts, 2000). Although it is generally thought of as a disease of women, men do contract breast cancer with a frequency of 1% of that in females (Giuliano et al., 1998; Giuliano et al., 2001).

Recent data have shown that mortality rates had significantly declined during the 1992-1996 period (Ries et al., 1999), probably as a result of earlier detection and improved treatments, and that the largest decreases occurred in younger women of both whites and African Americans. Some 40,800 deaths in women (and 400 deaths in men) were estimated to occur in 2000 in the U.S. (Cancer Facts, 2000). The 5-year relative survival rate for
localized breast cancer has increased 24% (from 72% to 96%) during the last 50 years. For regionally spread cancer the 5-year relative survival rate is 77%, and for patients with distal metastases the rate is 21%. Long-term survival after a diagnosis of breast cancer continues to decline as well beyond 5-years, where 71% of women diagnosed with breast cancer survive 10 years, and 57% survive 15 years (Cancer Facts, 2000).

1.2.2. Risk Factors.

Mammary carcinogenesis is a complex process, and the precise causes are not known with certainty. The risk of breast cancer seems to depend on a complex of familial, hormonal, and environmental factors, as the evidence, coming predominantly from epidemiological studies, has suggested that breast cancer may be multi-etiological in nature (Rebbeck et al., 1997). The evidence is derived from studies of geographic variations and effects of migration on breast cancer incidences (Adams et al., 1992; Ambrosone et al., 1997); of the time trends in cancer incidence and mortality (Ries et al., 1999); of demographic and socioeconomic factors (Ambrosone et al., 1997); of hereditary and genetic predisposition factors which influence individual susceptibility (Lancaster et al., 1997; Gorski et al., 1997); of the characterization of putative causative factors such as contraceptives and hormone replacement therapy usage (Hulka et al., 1997), exposure to certain chemicals or mixture of chemicals present in various occupational and environmental sources as well as endogenous substances, and timing of exposure to these substances (Eldridge et al., 1997; Safe et al., 1998), radiation exposure (Land et al., 1997), dietary factors (Rose et al., 1997), and oxidative damage as a result of endogenous metabolic reactions that generate highly reactive forms of oxygen (Cerutti et al., 1991; Pryor et al.,
The extent to which oxidative stress contribute to human cancer is not fully known. However, the production of various species of activated oxygen can enhance carcinogenesis in experimental systems by producing oxidative damage to DNA or cellular membranes and proteins, thereby causing mutations and/or altered products which may enhance cell proliferation and transformation (Cerutti et al., 1991; Pryor et al., 1993; Kubes et al., 2000; McCord, 2000). Recent studies have implicated reactive oxygen intermediates in activation of a variety of kinases such as the Src kinase family, protein kinase C (PKC), mitogen-activated protein kinase (MAPK), receptor tyrosine kinases, and transcription factors such as AP-1 and NF_{k}B (McCord, 2000). Although specific viruses such as hepatitis B, Epstein-Barr, papillomas, and some retroviruses have been linked to different types of human cancers, and the mice mammary tumor virus (MMTV) model has been used experimentally, the role of viruses in human breast cancer is not yet clarified (Trichopoulos et al., 1996).

Several risk factors, however, seem to be associated with steroid sex hormones exposure, i.e., reflecting the effects of circulating hormones levels throughout life (Gorski et al., 1997). It has been estimated that 40-60% of human cancers may be etiologically associated with sex hormones' exposure, either endogenous or exogenous (Li et al., 1991).

The risk of breast cancer increases with age, which appears to be a strong risk factor in view of the reports that breast cancer is predominantly postmenopausal in onset, i.e., much of the risk of developing (and dying of) breast cancer is incurred after age 75 (Dickson et al., 1995; Beers et al., 1999). The risk is higher in women who have personal or family history of breast cancer; those who have a biopsy-confirmed atypical hyperplasia; and those who
have a long menstrual history, i.e., early puberty and late menopause are putative risk factors, whereas loss of ovarian functions early in life is considered protective. The higher risk is also associated with induced abortion, and with those who have never had children or had their first child after the age of 35. Thus full-term pregnancy is protective early in life, but again becomes a risk factor after occurring for the first time at approximately 35 to 50 years of age (Rosner et al., 1994). Although most breast cancer cases are sporadic, approximately 10 percent are familial, with perhaps 1 percent considered being hereditary (Hulka et al., 1997). Hereditary breast cancer is characterized by a very high incidence in association with other cancers, premenopausal onset, excess bilateral disease, and multiple primary tumors (Dickson et al., 1995).

The possible risks associated with usage of oral contraceptives (OCs) or postmenopausal hormone replacement therapy (HRT) have represented a topic of controversy. The reported risk is higher in women who have recent use of OCs or HRT (Beck et al., 1994), and the excess risk of OCs use was reported to drop rapidly after the drug is stopped, suggesting late-stage tumor promoting effects (Harris JR et al., 1992). The risk of breast cancer was reported to be higher in women who consume two or more drinks of alcohol daily (Nestle et al., 1999). The prepubertal and pubertal exposure to ionizing radiation are also associated with higher risk of breast cancer (Harris CC et al., 1992; Dickson et al., 1995). Increased risk in women is also associated with higher education and higher socioeconomic status, probably due to other confounding factors associated with these statuses (Dickson et al., 1995; El-Bayoumy et al., 1997).

Several epidemiological studies have suggested dietary influences, with a high intake
of fat to be a possible major risk factor because of its association with serum estrogen level, and synthesis of other active substances such as eicosanoids (Dickson et al., 1995; Rose et al., 1997). However, precise causal roles for dietary factors have not been firmly established (Harris JR et al., 1992). Internationally, breast cancer prevalence is higher in the western industrialized countries, including the U.S., than the oriental and developing countries, where breast cancer incidence rates appear to correlate with variations in diet, particularly fat intakes (Prentice et al., 1990; Rose et al., 1997). The presence of BRCA1 and BRCA2 susceptibility genes, weight gains, physical inactivity, and exposures to pesticides and other chemicals are also associated with increased risk of breast cancer (Lancaster et al., 1997; Cleary et al., 1997; Safe et al., 1997; 1998).

1.2.3. Clinical presentation. Pathology, and Detection of Breast Cancer.

Breast cancer is usually presented as a postmenopausal disease, and it can be of particularly a poor prognosis when premenopausal (Dickson et al., 1995). The disease is usually detected as a mass by clinical or self-examination or mammography. Very small lesions are detected based on the appearance in mammography of microcalcifications in the tumor area (Bassett et al., 1997). It has been estimated that from the time of the earliest possible detection, a 1 cm³ tumor mass may require up to eight years to grow, and during this preclinical period the tumor may metastasize to distant sites, where a single metastatic site may contain up to $10^9$ cells (Dickson et al., 1995). Metastases may be detected initially in the regional lymph nodes prior to spread to distant sites such as lung, brain and bone.

Pathologic analyses had allowed the description of breast tumors as intraductal, locally invasive, or metastatic. The disease is staged as I-IV, depending on primary tumor
size, lymph node involvement and combined histologic grading. Stages I and II are intraductal, III is locally invasive, and IV is more widely metastatic disease (Giuliano et al., 1998). A complicated complex frequently described under the catchall name fibrocystic disease is considered to be relatively nonmalignant. The conditions termed hyperplasia, papilloma, and sclerosing adenosis are observed to have 1.5 - 2 fold increased risk of cancer, and the condition of atypical hyperplasia is reported to have 4 - 5 fold increased risk (Harris JR et al., 1992; Dupont et al., 1992). Atypical hyperplasia plus positive family history of breast cancer in first-degree relatives was reported to increase the risk by nearly nine folds (Beers et al., 1999). Carcinomas in situ (CIS), a cancer of either the mammary secretory lobules (LCIS) or ductules (DCIS), is considered to carry 8- to 10- folds increased risk of invasive breast cancer (Dickson et al., 1995). The CIS was reported to account for more than 15% of all breast cancers diagnosed in the USA (Beers et al., 1999).

Numerous studies have shown that early detection increases survival and treatment options. For women aged 20-39 it is recommended to have a clinical breast examination by a health care professional every three years and should perform monthly breast self-examination. All suspicious lumps or areas should be biopsied for a definitive diagnosis (Giuliano et al., 1998). Although there is still no consensus, even controversy exists, about the cutoff age to start annual mammography, it is recommended that women age 40 and older should have an annual mammogram, in addition to the annual clinical breast examination by a health care professional and the monthly breast self-examination. (Bassett et al., 1998). The decline in breast cancer mortality has been attributed, in part, to the use of regular screening mammography.
1.2.4. Treatment, Prognosis, and Prevention.

Considering the medical circumstances and preferences of the patient, treatment in general may include two or more of the following modalities: surgery by lumpectomy and removal of the regional lymph nodes, or mastectomy and removal of the regional lymph nodes; radiation therapy; chemotherapy in both hormone-dependent and -independent disease; and hormone therapy including high doses of estrogen or progestin, luteinizing hormone-releasing hormone (LHRH) agonists, antiprogestin, and antiestrogen drugs such as tamoxifen which has become a mainstay of hormonal therapy in hormone-dependent disease (Giuliano et al., 2001). Hormonal responsiveness was reported to be evident in about one third of breast cancers by the time they are metastatic (Dickson et al., 1995).

Treatment of stages I and II breast cancers with surgery and local radiation were reported to be highly successful. Treatment of the metastatic disease, however, requires the addition of combination of cytotoxic chemotherapeutic drugs and anti hormonal drugs as adjuvant therapy. The antitumor drugs are also used preoperatively, as neoadjuvant therapy, to shrink the tumor size (Giuliano et al., 1998). The use of the selective estrogen-receptor modulators (SERMs) such as tamoxifen and raloxifene was shown to be associated with decreased risk of breast cancer in several studies, however, there was a concomitant increased risk of uterine cancer reported with the usage of these agents (Giuliano et al., 2001). The immunotherapy of breast cancer has not yet been a particularly promising avenue of investigation (Giuliano et al., 2001).

The prognosis of breast cancer and response to therapy depends on special characteristics of the condition. Large tumor size, increased number of lymph nodes
involvement, and distant dissemination are associated with poor prognoses. Absence of estrogen receptors (ER) and the estrogen-inducible progesterone receptors (PR), poor nuclear grade, high DNA content (ploidy), high proliferative indexes, and the presence of an amplified oncogene such as c-erbB2 were also associated with poor prognoses (Dickson \textit{et al.}, 1995; Giuliano \textit{et al.}, 2001). Several studies have reported that for early stage breast cancer, long-term survival rates after lumpectomy plus radiation therapy, are similar to survival rates after a modified radical mastectomy. The advances in breast reconstruction techniques, which could be done at the same time as mastectomy, provide other options for breast reconstruction after mastectomies (Giuliano \textit{et al.}, 1998).

The high cost of breast cancer, emotionally, physically and financially, to the individual and society at large, necessitate the adoption of preventive strategies such as chemoprevention, life style changes, and early detection and treatment. Chemoprevention in particular is a promising and efficient approach to breast cancer prevention, and clinical trials of different phases are underway using different agents, such as synthetic retinoids and tamoxifen prophylactically in attempt to decrease incidence of breast cancer in women at high risk.

\textbf{1.3. Natural History of Mammary Carcinogenesis.}

Despite the multitude of risk factors including environmental, hormonal, behavioral and inheritance that have been linked to breast cancer, a unifying theory of mammary carcinogenesis remains elusive (Lancaster \textit{et al.}, 1997).
1.3.1. Normal Breast Development.

Examining the normal developmental stages of the mammary gland provides important insights into the factors and mechanisms that may be involved in mammary carcinogenesis. Indeed, most of the regulated or programmed expression (induction/repression) of the group of genes and proteins involved in cellular proliferation and differentiation, occurs normally during development, in embryogenesis, wound healing and cellular migration. It is the unregulated expression of these programs that appears to lead to the multiple phenotypes associated with the proliferative, invasive, and metastatic cancer.

**Development.** Early in human fetal life, epithelial cells (called an epithelial rudiment) derived from the epidermis proliferate into the underlying mesenchyme (the fatty stroma), and the formed short cords later develop luminae to become ducts that are connected to the nipple and open to the surface (Frantz et al., 1998). The mechanisms underlying the initial ductal penetration of the underlying fat are poorly understood. In the later stages of gestation the blind club-shaped terminal ends of these ducts undergo budding to form alveolar structures with a minimal amount of secretory activity, and by the end of gestation - with the decline in circulating fetal and placental hormones - the fetus breast regresses to a resting stage composed of a small number of scattered ducts (Frantz et al., 1998; Russo et al., 2001). At puberty and shortly before menarche, under the positive regulation by estrogen and pituitary growth hormone (GH), or its local mediator, IGF-I, glandular changes begin in the form of lengthening and branching of the ducts that are accompanied by budding of the terminal ends (called terminal end buds, TEBs); and stromal changes occur in the form of increased formation of underlying fat and connective tissue. With the onset of menses,
Further growth takes place in a cyclic fashion, with regression occurring at the end of each cycle (Dickson et al., 1995; Frantz et al., 1998; Russo et al., 2001). The TEBs give origin to new branches and finally to alveolar buds (or ductules), and the alveolar buds cluster around a terminal duct forming a lobule (Russo et al., 2001). The lobule formation in the female breast occurs within 1-2 years after the onset of the first menstrual period, but full differentiation of the gland structure is a gradual process that may take years, and in some cases if pregnancy does not supervene, may be never attained (Russo et al., 2001). Thelarche (defined as the initial clinical appearance of the breast bud) occurs at an average age of 11.2 years (± 0.7 SD) in white females, and one year earlier in African-American females (Russo et al., 1992). During pregnancy, the lobulo-alveolar maturation into acini (the terminal structure of the mature gland) occurs under the influence of several hormones, as the mammary epithelium proliferates and differentiates into a secretory epithelium committed to the synthesis of milk, a rich source of growth factors that may further play an important role in mammary differentiation (Dickson et al., 1995; Schedin et al., 1996). Withdrawal of the lactogenic hormones' stimulation (following weaning) has been observed to lead to a complex process of mammary involution characterized by tissue remodeling and apoptosis in the mammary epithelium (Dickson et al., 1995; Schedin et al., 1996), which may result in elimination of a portion of potentially deleterious cells that might be more susceptible to acquire or accumulate alterations and therefore at higher risk of developing neoplasia (Schedin et al., 1996).

**Differentiation Stages.** Studies of the human adult normal breast tissue had identified at least four types of lobules, designated type-1, -2, -3, and -4 lobules, and the transition from
type-1 to the more mature types-2 and -3 represent a gradual process with increased number of new alveolar buds (or ductules) (Russo et al., 2001). In nulliparous women, independent of age, breast tissue was reported to contain more undifferentiated structures (Russo et al., 1992) and this pattern remains constant throughout the reproductive life span unless pregnancy ensues; whereas in parous women, the predominant structures observed were the more differentiated lobule types (Russo et al., 2001). It was reported that a history of parity between the ages of 14-20 years correlates with a significant increase in the number of type-3 lobules, which remain present as the predominant structure until after the age of 40 years (Russo et al., 1992). Type-3 lobules associated with pregnancy were observed to develop into fully differentiated type-4 lobules during lactation, after which they regressed to type-3 lobules during post lactation involution (Russo et al., 1992). After the menopause, which is estimated to occurs at an average age of 51 years, the breast was observed to regress in both parous and nulliparous women (Russo et al., 2001). Thus, the overall progression/regression process differs between nulliparous and parous women, where early parous women truly undergo lobule differentiation evidently at a younger age, whereas the nulliparous women seldom reached type-3 lobule stage, and never reached the type-4 lobule stage (Russo et al., 2001).

Studies of the proliferative activity of the mammary epithelium in both rodent and humans have demonstrated that cell division varies with the degree of differentiation of the mammary parenchyma (epithelium) (Russo & Romero, 1994; Russo & Russo, 1, 1997). In women, the highest level of cell proliferation, reflected in the proliferative index (PI), was observed in the undifferentiated type-1 lobules, and the progressive differentiation into
higher types of lobules has resulted in concomitant reduction of the proliferative activity (Russo et al., 1992; 1996; 2001).

Recent studies of hormone-receptor contents have shown that estrogen receptor α (ERα) and progesterone receptor (PR) contents in the lobular structures of the breast are directly proportional to the rate of cell proliferation, and inversely proportional to the degree of differentiation, that is, the receptor content and proliferation are maximal in the undifferentiated type-1 lobules, and decrease progressively in types-2 (three fold lower in PI), and type-3 (10 fold lower in PI) and -4 (Russo et al., 2001). The findings that cells with the highest proliferative activity and receptor content occur in type-1 lobules (compared to types-2 and -3) provide support to the notion that type-1 lobules are the sites of origin of ductal carcinomas (Russo et al., 1992), and it may also provide a mechanistic explanation for the higher susceptibility of these structures to be transformed in vitro by chemical carcinogens (Russo et al., 1993). However, examining the proliferative activity and the receptors content simultaneously has revealed new findings. It was observed that proliferating human breast cells differ from those that contain hormone receptors and their co-activators (Clarke et al., 1997; Russo et al., 2001), that is, the cells that are proliferating are not necessarily the same cells which are (ER+) or (PR+). A few cells in type-1 lobules showed increased PI plus (ER+) or increased PI plus (PR+) reactivity, with even more negligible numbers in types-2 and -3 lobules (Russo et al., 2001). These findings, which suggest that the proliferating cells and (ER+) cells are two separate populations, support the notion that estrogens may control cell proliferation by an indirect (paracrine) mechanism (Clark et al., 1992; Russo et al., 2001), that is thought to mediate the actions of antiestrogens as well (Parker, 1996). These
findings were supported by in vitro results which have shown type-1 lobules of normal breast tissue to lose the (ER+) cells when placed in culture, suggesting that only proliferating cells which are (ER-) can survive and that those cells may constitute the stem cells (Pilat et al., 1996; Russo et al., 2001). Such findings, therefore, suggest that the mammary gland may respond selectively to hormonal stimuli depending on specific topographic differences in the gland development which influence the expression of either proliferation or differentiation (Russo et al., 2001).

Hormones. The regulation of normal breast development, proliferation and differentiation, as well as the development, growth and progression of breast cancer appear to be dependent on hormonal factors including endocrine steroids (estrogen, progesterone, corticosteroids), and endocrine peptides (prolactin, thyroid hormone, growth hormone, insulin) (Frantz et al., 1998; Russo et al., 2001; Tripathy et al., 2001). In addition, normal and malignant mammary tissues are able to synthesize locally acting hormone-like molecules, such as polypeptide growth factors, prostaglandins, and fatty acids that may act in a paracrine or autocrine fashion (Dichson et al., 1995).

In the non-pregnant female the normal development of the mammary gland is rigorously controlled by the ovarian estrogen and progesterone. Prolactin is reported to play an additional role, but its specific actions are not fully understood (Russo et al., 2001). Clinical studies have defined the relationship between mammary epithelium and ovarian hormones' production in different phases of the menstrual cycle. In non-pregnant women, the cyclicity of the reproductive hormones leads to parallel cyclicity in the mammary gland growth pattern, in contrast to that observed in the endometrium (Dickson et al., 1995).
breast epithelium does not exhibit maximal proliferation during the follicular phase of the menstrual cycle, but the maximal proliferation occurs in the luteal phase, after which the cessation of proliferation (brought by the drop in hormones level) is followed by apoptosis (Goodman et al., 1994; Laidlaw et al., 1995; Russo et al., 1996; Clark et al., 1997).

Although the breast is influenced by a myriad of hormones and growth factors, estrogens are considered to play a major role in promoting the proliferation of both the normal and malignant breast epithelium (King et al., 1992; Russo & Russo, II, 1997; Russo et al., 1996; 1998; 2001). It was reported that breast cells grown in vitro or when breast tissues are implanted in athymic nude mice, estrogens alone stimulate cell proliferation, and progesterone has no effect or even inhibits cell growth (Laidlaw et al., 1995; Clarke et al., 1997). Experimental studies on mammary cell lines have suggested that estrogen may exert its proliferative activity on mammary epithelium by at least three mechanisms: direct receptor-mediated stimulation (Watts et al., 1992); indirect stimulation; or indirect inhibition of inhibitory factors present in the serum (Russo et al., 1996). The precise roles of these mechanisms in the normal development and differentiation of the breast, or in the initiation, promotion, and progression of breast cancer have not yet been fully defined. Adding to the complexity is the discovery of a second type of estrogen receptor (ER-β), a genetically distinct estrogen receptor, which is reported to be present in human, mouse and rat, and has an affinity for estradiol similar to ER-α (the classical estrogen receptor), however, they display nonidentical expression patterns in target tissues (Hall et al., 2000; Russo et al., 2001). In addition, ER and PR-mediated transcriptions often involve binding of co-activator (such as SRC-1) to the transcription complex to enhance the receptors'
function, and SRC-1 gene knockout was observed to decrease the growth and development of mammary gland (Xu et al., 1998). However, the essential roles of ER-α and PR (as indicators of hormonal effects) in mammary gland development have been demonstrated by studies in knockout mice lacking functional receptors. The ER-α knockout mice displayed grossly impaired ductal epithelial proliferation and branching, while PR knockout mice displayed significant ductal development but decreased arborization and absence of alveolar differentiation (Lubahn et al., 1993; Bocchinfuso et al., 1997).

**Stromal Effects.** The epithelial-stromal interactions may play important roles in human breast development, particularly after the demonstration that stromal tissue can synthesize estrogens through the aromatase enzyme, and this locally produced estrogens may act in a paracrine fashion (Santner et al., 1997; Cunha et al., 1996; 1997). This is also consistent with the findings in the uterus and prostate, where the stroma was found to be necessary for epithelial proliferation (Cooke et al., 1997; Hayward et al., 1997). Also indicative of interaction is the reported localization of SRC-1 in rat mammary gland which was observed to differ between epithelial and stromal tissues, as SRC-1 appears to be segregated in distinct subset of cells within (ER+) epithelial cells’ population, in contrast to the stroma where significant number of cells co-expressed both ER-α and SRC-1 (Russo et al., 2001). Another epithelial-stromal interaction-related factor, that may influence mammary epithelium proliferation and differentiation, is the basement membrane which is reported to exert an organizing effects on mammary differentiation (Monteagudo et al., 1990; Streuli et al., 1998).
1.3.2. Modeling of Breast Carcinogenesis.

The traditional view of carcinogenesis is derived primarily from studies of experimental models, and a currently accepted view of cancer development and growth has proposed carcinogenesis to be a **monoclonal multi steps** process (Buick *et al.*, 1992).

The *Monoclonal Origin Concept* (as opposed to polyclonal with multiple origins) proposes that tumor arises from a single altered cell whose progeny will constitute the tumor mass. This concept is supported by the evidence that uniquely identifying features (i.e. clonal markers) can be found in all the cells of many types of tumors (Wainscoat *et al.*, 1990; Bishop, 1995). It is worthy to note that an important property of clonal expansion - *that there is an inverse relationship between cellular differentiation and proliferation potential* - where the more mature cells may continue to differentiate without proliferation and are ultimately lost from the population as several homeostatic mechanisms maintain the balance between cell production and cell loss (Buick *et al.*, 1992; Tannock, 1992), has led some investigators to related monoclonality to the concept of stem cell in tissue renewal, and tumorigenesis in this view is thought of as aberrant cell renewal where the changes associated with the carcinogenic insult may produce defects in the control of the normal stem cell functions (Buick *et al.*, 1992). This view has maintained that the properties of stem cells are such that it is not necessary to assume dedifferentiation as a mechanism of tumor induction (Pierce *et al.*, 1988). An alternative theory has suggested that carcinogenic events might occur in a normal differentiated cell, rendering that cell proliferative although retaining to some extent its ability to organize tissue-specific differentiation, however, the acquisition of proliferative features in a differentiated cell would necessitate the concept of
dedifferentiation (Buick et al., 1992). Several studies have provided evidence in support of the validity of the stem cell mechanism in the development of human tumors (Daniel et al., 1987; Pierce et al., 1988; Buick et al., 1992). The stem cell concept has an important implication for the treatment of human cancers and underscore the importance of cancer prevention. Since the aim of cancer therapy is cure, or at least long-term control, the therapy must be directed toward eradication of the altered stem cells which have the potential to regenerate the tumor population.

As evident from the risk factors profile of breast cancer, the Multi Steps Concept proposes the development of a fully malignant tumor to involve complex interactions over time between several factors, both exogenous (environmental) and endogenous (genetic, hormonal, immunologic) that produce multiple disturbances in cellular mechanisms and gene expressions. In other words, the multi stages’ carcinogenic process involves the progressive acquisition of genetic as well as epigenetic abnormalities in the expression of multiple genes that have highly diverse functions (Weinstein, 2000). The multistages concept, which implies multiple causative factors, multiple cellular targets, multiple biochemical reactions, and multiple stages of evolution, could be illustrated through the “initiation/ promotion / progression model” of carcinogenesis. This model had originated and is often associated with chemically-induced carcinogenesis, as several experimental studies have suggested that induction of cancer in different organs and tissues involves processes analogous to the two stages (initiation/ promotion) model of mouse skin cancer (Archer, 1992; Weinstein et al., 1995). The multi phases model, a time line-related model, basically expands on the “sequential multi hit model” by proposing that carcinogenesis may proceed through
discernible phases, and that the transition between these stages may probably be driven by multiple factors and involves different cellular elements and mechanisms. Most important, this model suggests that the transition between successive stages can be enhanced or inhibited by different agents (Bishop, 1995; Weinstein et al., 1995). Although neither the specific etiological agent(s) nor the precise mechanism(s) responsible for human breast cancer causation and initiation have been yet clearly identified (Russo et al., 1994), this model might be of help in devising strategies for the prevention and therapy of this disease.

Figure (1.1) illustrates a general scheme of the multistages carcinogenic process that might occur in mammary tissues. The demarcation of the stages, that is, their boundaries are not definitely determined by any means and should rather be viewed as overlapping on the time line course. It seems from the weak association of the fibrocystic complex (except atypical hyperplasia) and breast cancer risk that breast cancer does not always evolve through well defined morphological stages; and it has not been possible to establish the order in which cellular alterations occur in this type of tumors (Russo & Russo, II, 1997). It should also be noted that although in some experimental models the stages can be clearly defined, in the real world humans often are exposed repetitively and may be simultaneously to different putative initiating and promoting agents. Therefore, there may be repetitive cycles of cellular alterations, tumor promotion, and clonal expansion.
Figure 1.1: A Schematic Description of Multi-Phase Carcinogenesis.

--- denote inhibition / arrest; ----> denote stimulation / progression.
The "initiation / promotion / progression model" simply postulates that the time between exposure to a known or putative carcinogen and the clinical detection of tumors may be divided into at least three distinct phases: initiation (which ends with the development of initiated cell), promotion (which ends with the development of benign or premalignant lesions), and progression (the conversion of premalignant to malignant lesions and their further evolution to tumors with increasing degree of malignancy) (Weinstein, 1988; Harris JR, 1992; Sugimura et al., 1992; Castagna et al., 1992; Vogelstein et al., 1993).

The first phase, initiation, may result from exposure to carcinogenic stimuli, and the initiating agent(s), whether exogenous or endogenous, may affect different cellular molecules such as proteins and nucleic acids at numerous sites within the cell resulting in their alteration (aberrant activation), and they may act either directly on these cellular molecules, or indirectly by producing such agents as free radicals or through activation of growth stimulatory pathways (Archer, 1992; Weinstein et al., 1995; 2000). It was reported that, at this stage, the initiated cells cannot be distinguished from other cells of the affected tissue, and little is known as yet about their genotypic or phenotypic properties, except that they are susceptible to stimulation by promoting agents to develop focally into discrete preneoplastic then neoplastic lesions. Also the exact number of alterations, and whether they occur first at certain genes (e.g. oncogenes or tumor suppressor genes) are not precisely known (Archer, 1992; Weinstein et al., 1995). In the chemically-induced rat mammary tumors, there is evidence that activation of individual members of the ras family of proto-oncogenes may be the initiating event in carcinogenesis, however, ras products are also associated with promotion and progression (Archer, 1992; Minden et al., 1992). It should be noted that the
initiated cells are not yet tumor cells in themselves since they have not yet acquired the autonomy of growth (Archer, 1992).

The second phase, promotion, is proposed to start with the initiated cell, and ends with benign or preneoplastic cells that are immortalized. Promotion, under the effects of promoting agent(s) during the long latency period before the development of the first transformed cancer cells, appears to involve a series of cellular and tissue changes that produce persistently aberrant cells with autonomy of growth potential (Archer, 1992; Weinstein et al., 1995). There is as yet no unifying principle to explain the diverse activities of putative promoting agents; and their mechanisms of action to enhance the growth of the altered cells are poorly understood. Studies of established promoting agents such as phorbol esters, have shown that they produce pleiotropic effects including changes in the phospholipid synthesis, DNA and RNA synthesis, enzyme induction, receptor expression, polyamide synthesis, and prostaglandin release, with concomitant changes in mitotic rate, cellular morphology, and degree of terminal differentiation. However, none of these various effects can be identified clearly as the one critical for tumor promotion (Archer, 1992; Weinstein et al., 1995). Although some evidence has suggested that a primary targets of these agents may be cell membranes, other studies have suggested that part of the promoting agents' cellular effects may be produced by modulating the expression of other cellular genes that have complementary functions in the initiated cell, and by stimulating the activity of cytoplasmic protein kinases such as protein kinase C , which is implicated as a second messenger in the action of several hormones and growth factors that functions through membrane receptors (Archer, 1992; Sutherland et al., 1992; Minden et al., 1992; Weinstein
It is not yet fully clear whether the only effect of promoters is the stimulation of initiated cells' selective proliferation, or whether they cause cellular damage as well, since some experimental studies have suggested that some promoters can produce cellular damage through the production of activated forms of oxygen (Perrera et al., 2000).

The third phase, progression, is proposed to start with the immortalized premalignant cells that would be malignantly transformed, and ends with the clinical picture of fully malignant and/or metastasized cancer. Several types of changes were observed to occur when a cell become malignantly transformed as compared to morphological transformation (Buick et al., 1992). In addition to immortalization (which appears to be a necessary but not a sufficient step in transformation to a malignant state), autonomy (loss of contact inhibition, anchorage independence, loss of dependence on serum and growth factors), extensive morphological and functional loss of differentiation, tumorigenicity, metastasis, and death if inhibited from growing were also observed changes (Lewin, 1997; Buick et al., 1992).

Thus, progression (the conversion of premalignant cells to fully malignant cells) seems to involve stepwise evolution of tumor cells as they become progressively more malignant cells that display increasing heterogeneity, autonomy, and drug resistance. The factors and mechanisms that mediate tumor progression are poorly understood. However, the development of subclones with different properties is thought to be a major mechanism underlying this process (Waghorne et al., 1988; Miller et al., 1988). It was postulated that the acquired heterogeneity within the original clone of the cancer cells may allow sequential selection of more aggressive (in terms of invasiveness, metastasis, and drug resistance)
subclones (Buick et al., 1992). A proposed model have suggested that tumor cells tend to
be genetically unstable and therefore subject to a higher rate of random mutations during
clonal expansion from a single transformed cell of origin. A few of these alterations may
convey greater autonomy and selective growth advantage, and those more malignant
subclones will tend to become dominant in the tumor population, leading to tumor
progression (Nowell, 1986). In regard to tumor progression, it is worthy to note that some
malignant cell lines can be rendered nonmalignant by agents such as dimethyl sulfoxide
(DMSO), 5-azacytidine, and retinoic acid which promote cellular differentiation in tissue
cultures, and a reported common feature of these differentiated cells was the induction of
gene expressions that would have been expected in normal differentiated cells of the same
lineage (Buick et al., 1992).

Some of the lingering difficulties with the multistages model of carcinogenesis have
risen primarily from attempts to define and categorized the myriad of putative carcinogenic
agents and mechanisms involved in such complex process into well-demarcated areas of
coupled carcinogens/ mutagens, initiators/ initiation, promotors/ promotion and so forth.
Added to the difficulties are the initial characterizations of terms such as carcinogen,
mutagen, initiator, promotor, necessary or sufficient agent, their relationship to each other,
and their relationship to the model itself, which were not adequately explained. For instance,
restricting the mechanisms of action of initiator(s) to genetic mutations only may appear
contradictory to the notion of occurrence of subsequent mutations that are assumed to play
a significant role in the consequent steps of the carcinogenic process (Fearon et al., 1990;
Sugimura et al., 1992; Benchimol et al., 1992; Howley et al., 1995; Weinstein et al., 1995).
Thus, an initiator(s) may act by genetic or epigenetic mechanisms (Weinstein, 2000). In addition, many of the substances that are carcinogenic in rodent tests did not score as mutagens in the short term tests (Harris CC, 1991; Bishop, 1995), therefore, a carcinogen may or may not be a mutagen. The observation that some chemical carcinogens, apparently, have the ability to act alone to cause cancer after administration in animal models suggests that some agents can act as both initiator(s) and promotor(s) (Archer, 1992). Also, the observation that viral agents by themselves are not considered sufficient for induction of associated neoplasia, since co-factors are believed to be important in viral-induced cancers (Howley et al., 1995), suggests that some mutagenic agents are necessary but not sufficient for the induction of carcinogenesis. In addition, inter-individual variations (polymorphism) in a carcinogen metabolism may affect the carcinogenic outcome (Archer, 1992; Rothman et al., 2001).

Similar logic can also be applied to the characterization of a promotor, which is classically described as an agent that possess little or no carcinogenic potency when tested alone, but enhance clonal proliferation when present continuously following the initial (usually proposed as DNA damaging) carcinogenic event (Weinstein et al., 1995). Such characterization does not fully account for a promotor's independent (from initiator) effects and varied functions. Although promotors appear to act largely at the epigenetic level by altering signal transduction pathways, gene expression, and cellular differentiation, thus enhancing cell proliferation and clonal expansion of previously initiated cells (Weinstein et al., 1995), some of these mechanisms may have been already in effect before the exposure to the putative initiator(s) (Iversen, 1993). In addition, promotors may contribute directly
(oxidative damage) or indirectly (enhanced proliferation) to the development of genetic (DNA) damage (Perera et al., 2000).

Thus, it seems that redefining the classical characterization of initiation/promotion model of chemically-induced animal carcinogenesis may help to adequately represent human cancers. The decoupling of such terms as carcinogen/mutagen, initiator/initiation, and promotor/promotion, along with reconsidering the multi phases process as levels with qualitative and quantitative characteristics may simplify the application of this model to the complex process of carcinogenesis and increase its relevance to prevention strategies. The initiation phase can be qualitatively conceptualized as the primary level, where the less-differentiated proliferating cells are more prone to respond to proliferating stimuli by initiating agent(s), whether the activation of such stimulatory pathways occurred by genetic (mutational) or epigenetic mechanisms. The initiated cells, with its acquired enhanced proliferation potential, may then proceed to the second level, the promotion phase where promotors - a multitude of agents (that may also include the initiating agents) - act by genetic and epigenetic mechanisms to enhance proliferation and induce the qualitative acquisition of more aberrations needed for the establishment of premalignant tumor cells. In the next progression phase, those and additional agents/mechanisms may induce the occurrence of malignant transformation. The sub-colony developed may continue to acquire more alterations needed for invasiveness and metastasis. Quantitatively, there are differences in the number and intensity of alterations that occurs in these consequent phases. Finally these levels could be viewed as parallels to the primary (susceptibility), secondary, and tertiary levels of prevention.

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1.3.3. Breast Cancer Development.

The pathogenesis of breast cancer is not yet fully known, mainly due to the long latency period between initiation and tumor detection, the lack of identification of a specific site in which the initial events occurred, or the timing of its initiation (Russo & Russo, II, 1997). Although breast cancer can be characterized by dysregulated proliferation and loss of differentiation properties, the precise mechanisms that underlie the excessive proliferation, loss of normal organization (morphological differentiation) and certain epithelial characteristics (functional differentiation), as well as loss of compartmentation (metastasis) are not known with certainty. However, the growth and progression of breast cancer appear to be dependent on interactions between hormones, hormone-like factors (e.g. fatty acids, prostaglandins), growth-modulating gene products, and nuclear factors, in a specific less differentiated compartment of the breast (Dickson et al., 1995; Russo & Russo, I, 1997).

It is not yet known when in the lifetime of a women the initiation of breast cancer takes place, or whether specific agent(s) cause it, however epidemiologic and laboratory studies have suggested that the period between menarche and first full-term pregnancy may represent a window of high susceptibility for the initiation of breast cancer, when the breast developmental structure is in its early stages (Russo & Russo, I, 1997). Further supportive evidence for this proposal comes from studies of prophylactic mastectomy specimens obtained from women with familial breast and ovarian cancers or carriers of BRCA-1 gene, which have indicated that genetic determinants seem to influence the pattern of breast development and differentiation (Russo & Russo, II, 1997).
Mechanistic Issues in Mammary Carcinogenesis.

Several mechanisms are proposed to contribute to the carcinogenic process, as neoplastic growth - in general terms - appears to be related to aberrations in the control of cell proliferation and differentiation. Aberrations that are brought about by cellular instability as a result of carcinogenic injuries. Thus, tumor can grow by a process of clonal evolution driven by accumulation of genetic and epigenetic aberrations, particularly in expression of growth-regulatory genes (Archer, 1992; Vogelstein et al., 1993; Kinzler et al., 1996; Bishop, 1995; Weinberg, 1996; Perera et al., 2000). In this context, cellular instability relates basically to imbalance between activation (stimulation) or inactivation (disruption) of certain signaling pathways that are associated with certain phenotypic characteristics of proliferation and differentiation. Activation/deactivation of a gene product can perturb a cell by changing either the amount or the activity of that gene product, and consequently the pathway depending on it. Although DNA mutation is apparently an effective way to achieve this activated/inactivated state, it seems not to be the only way operating. For example, in human (ER-) breast cancer cell lines with no mutations within the ER gene, aberrant (hyper) methylation of the estrogen receptor (ER) gene was reported to inhibit ER transcription, and demethylation was shown to correlate with re-expression of the ER gene (Ferguson et al., 1995). Activation of a growth stimulatory pathway may be accomplished by induction of a silent growth-stimulatory gene expression, or overexpression of a stimulatory gene normal product, or over-activity of a stimulatory gene-altered product; whereas inactivation of inhibitory pathway may be accomplished by inhibition of growth suppressor gene normal product, or altering a suppressor gene protein product (Weinberg, 1996). For the
development of fully malignant tumor, it seems that multiple alterations are needed, i.e., to accumulate in several of the cell's proliferation/differentiation controlling genes and their products, and additional alterations (activation/inactivation) in other groups of genes may enable these cells to become invasive and capable of metastasizing to other sites in the body (Vogelstein et al., 1993; Weinberg, 1996; Kinzler et al., 1996).

In this context, genetic alterations, are broadly defined to include any permanent changes in DNA structure (change in the nucleotide sequence or arrangement of DNA in the genome such as point mutations, deletions, insertions, and translocations) (Squire et al., 1992), as well as changes in DNA content such as gene amplifications. On the other hand, epigenetic changes are broadly defined as changes that may result in different properties of a cell but that does not represent a change in genetic information (Lewin, I, 1997). Aberrant DNA methylation, oxidative damage and effects of metabolism of nitrogen oxide and nitrites, and activation of receptors, signal transduction elements and transcription factors are reported examples of epigenetic alteration mechanisms (Ferguson et al., 1995; Lewin, I, 1997; McCord, 2000; Perera et al., 2000; Weinstein, 2000).

**Hormones.** Ovarian estrogens were implicated as primary factors among the hormonal influences associated with mammary tumors development and growth (Dickson et al., 1995; Russo et al., 2001). On the other hand, progestins are generally associated with inhibition of mammary cancer (Safe, 1998), although the results from animal models were variable. In rodent models of chemically-induced mammary cancer, both estrogens and progesterone were shown to be able to support initial tumor formation and early tumor growth (Kordon et al., 1993; Dickson et al., 1995; Aldaz et al., 1996). However, it was also shown that the
synthetic progestin, MPA (medroxyprogesterone acetate) can inhibit carcinogen-induced mammary tumorigenesis (Dauvois et al., 1989; Li et al., 1995). The mechanisms of interaction between estrogens and progestins in both normal and malignant breast seem to be varied and complex, and are yet to be clarified (Clarke et al., 1990; Dickson et al., 1995). However, one mechanism in breast cancer is proposed to be based on the requirement of estrogen to induce expression of progesterone receptor (PR), which was observed in breast cancer cell lines to be positively regulated by estrogen (Dickson et al., 1995).

The exact mechanisms of action of estrogen and progesterone are not yet fully understood. It is proposed, however, that these actions involve regulation of transcription of responsive genes, post-transcriptional regulation of mRNA stability, and regulation of mRNA to protein translation (Dickson et al., 1995). The estrogens and progestins were shown to exert their action through dimerization of their receptors, and some reports have suggested that phosphorylation of different sites of the estrogen and progesterone receptors (induced by other hormones, growth factors, dopamine agonists, and cAMP) may activate these steroid receptors (Fawell et al., 1990; Power et al., 1991). The estrogens were also reported to modulate transcription through receptor binding to AP-1 sites (Dickson et al., 1995; Parker et al., 1996). There are two known forms of estrogen receptor (ER), ER-α and ER-β (Hall et al., 2000; Russo et al., 2001); and there are also two known closely related isoforms of progesterone receptor (PR), PR A and PR B, which exhibit different activities in vitro, with PR A demonstrating dominant negative inhibitory effects on the activity of PR B and other nuclear receptors (Dickson et al., 1995; McGowan et al., 1999). In rodent and humans, ER and PR have been localized to a luminal subpopulation of ductal and lobular
epithelial cells, i.e., these receptors appears to be present in partially differentiated epithelium (Dickson et al., 1995; Russo et al., 2001). The regulation of the ER expression in human breast cancer is a very complex process which is subject to regulation by estrogen itself (Russo et al., 2001), and the auto-regulation of PR expression by progesterone was also demonstrated in breast cancer cell lines (Dickson et al., 1995).

The relationship between (ER+) and (ER-) breast cancers is unclear. The differences between these two conditions encompass different in vitro and in vivo proliferative and invasive rates, expression of certain growth factors' receptors and enzymes of drug metabolisms, morphology and other indicators of differentiation (Dickson et al., 1995). It was proposed that either (ER+) and (ER-) breast cancers are different entities arising from different cell types of tumor origin; or (ER+) breast cancer may be resulting from expression of ER during transformation of (ER-) cells, or alternatively (ER-) breast cancer can result from the loss of ability of some cells to synthesize ER during evolution of (ER+) cancer cells, that is, the ER status is derived from a stage of differentiation from a cancer precursor cell (Habel et al., 1993; Harlan et al., 1993; Dickson et al., 1995; Hu et al., 1998; Russo et al., 2001). Another possibility is that those cells previously considered being (ER-), that is, ER-α negative cells, might be ER-β positive cells (Kuiper et al., 1996; Byers et al., 1997).

An additional possibility is that, aside from the traditional view that estrogen act through its receptor to transcriptionally activate specific target genes, there is evidence that a membrane receptor coupled with alternative second-messenger signaling mechanisms may also be operational, which suggest that (ER-) cells found in human breast may respond to estrogen through this pathway (Aronica et al., 1994; Pappos et al., 1994).
A link between protein kinase C (PKC) and (ER+) or (ER-) statuses has been reported, where PKC has been implicated in down-regulation of ER, inactivation of ER functions, and independent induction of some of the estrogen-responsive genes during malignant progression (Dickson et al., 1995; Davidson et al., 1996). It has also been observed that PKC expression is elevated in drug resistance (ER-) breast cancer relative to (ER+) breast cancers, and the stimulation of (ER+) breast cancer tissue with phorbol esters (TPA or PMA), known activators of PKC, has led to down regulation of ER and destabilization of its mRNA, consistent with the loss of the receptor function (Tzukerman et al., 1991; Stoica et al., 2000).

The relationship between ER and PR statuses, and responsiveness to hormonal therapy is also complicated. Significant levels of ER have been detected in more than 60% of human breast cancer, however, only 60% of those (ER+) tumors respond to endocrine therapy. On the other hand, approximately 10% of (ER-) tumors respond to endocrine therapy. Similarly, 70% of (PR+), and approximately 30% of (PR-) tumors have been reported to respond to hormone therapy (Russo et al., 2001). The reasons for such discrepancies are not completely clear, and could be attributed to different factors including differential metabolism of the antiestrogens (e.g. tamoxifen), the presence of other types of ER, the effects of absence of co-activators, and the presence of defective ER (Dickson et al., 1995; Russo et al., 2001). Adding to the uncertainty is the observation that while ER expression is associated with cellular proliferation in (ER+) cell lines, transfected ER expression in (ER-) cell lines was observed to function in suppressing cell growth (Russo et al., 2001). The negative effect of estrogen on those (ER-) cells transfected with the ER can
be attributed, however, to interference with transcription factors that maintain the estrogen-independent growth (Touitou et al., 1991; Jiang et al., 1992; Zajchowski et al., 1993).

**Oncogenes and Tumor Suppressor Genes.** Aberrant activation/inactivation in certain classes of genes such as proto-oncogenes, tumor suppressor genes, and cyclin-related genes, which in their normal configuration coordinate the life cycle of the cell, are presumed to induce various aberrant mechanisms that interfere with normal cellular responses to growth stimulation or inhibition signals (Ronai et al., 1990; Herrlich et al., 1994; Weinberg, 1996).

The **Oncogenes** which promote cellular proliferation can generally be classified into:

1. Genes that code for growth factors or their receptors such as PDGF, TGF-α, EGF, RET (growth factor receptor), erb-B (a receptor involved in breast cancer); and erbB2 (also called p185, p185 erbB-2 and p185 HER-2 in humans, and p185 neu in rats, codes for a growth factor receptor, and is also involved in breast cancer).
2. Genes that code for cytoplasmic relays (second messengers) in the stimulatory signaling pathways such as members of the ras family of oncogenes. Activated ras was found in a wide variety of human malignancies, including breast cancer, and the forced overexpression of oncogenic ras was shown to cause malignant transformation in multiple cell types including mammary epithelial cells. The constitutive activation of this small GTPase was shown to result in activation of downstream signaling proteins, including Raf and the downstream MAP kinases, as well as Raf-independent signaling proteins, including Rho family proteins, thus modulating the expression of certain genes and ultimately contribute to cell proliferation and malignant transformation. Although overexpression of ras family of oncogenes was observed to be common in human breast cancers, ras mutations were seldom seen in human tumor cells,
whereas mutations are common in experimental rodent mammary tumors. (3) Genes that code for transcription factors, which activate expression of other growth promoting genes, such as members of the myc family of oncogenes (e.g. c-myc which is involved in breast cancer). (4) Genes that code for other types of growth stimulatory molecules such as Bcl-1 (also called PRAD1, and codes for cyclin D1, a stimulatory component of the cell cycle and is involved in breast cancer); Bcl-2 (codes for a protein that normally blocks apoptosis); and MDM2 (codes for an antagonist of the p53 tumor suppressor protein, and is involved in many cancers). The genes which are activated early in the process such as c-jun, c-fos, c-myc, and tumor necrosis factor-α (TNF-α) are collectively called “immediate early response genes” (Hurta et al., 1992; Minden et al., 1992; Rosen, 1995; Weinstein et al., 1995; Weinberg, 1996; Medina, 1996; Sheng et al., 1998). There is considerable evidence to support the notion of a causal link between the induction of oncogenes and the proliferative process, as cellular responses to mitogens are generally mediated by sequential activation of receptor tyrosine kinases, src, ras, and one or more of the mitogen-activated protein kinase (MAPK) pathways (Weinstein et al., 1995; Smith et al., 2000). The induction of MAPK activity was reported to be essential for induction of COX-2 that occurs after activation of the ras pathway, whether by activation of EGF receptor tyrosine kinase or by activation of mutant Ras (Sheng et al., 1998).

The principal alterations observed in human breast cancer include amplification or overexpression of c-myc, c-erbB2, EGFR, cyclin D1, and cyclin E (Dickson et al., 1995). EGFR was implicated in cellular transformation, and the absence (or low level) of ER expression in conjunction with overexpression of EGFR was often noted in breast cancer.
cell lines, suggesting a mechanistic link between upregulation of EGFR and hormone-independence (Klijn et al., 1992). The association with c-erbB2 is somewhat complicated. The exact functions of c-erbB2 protein, which has substantial homology to EGFR, in normal tissue are not yet clarified, and the exact role of c-erbB2 as either an oncogene or a differentiation-inducing factor remains unresolved, since in cancer cells overexpressing c-erb-B2 protein, some growth factors (dose-dependently) can modulate (stimulate/ inhibit) cellular proliferation and differentiation (Samanta et al., 1994; Dickson et al., 1995). Although amplification of c-erbB2 seems to occur in all stages of breast cancer (Moffett et al., 1992), experimental results of cell line transfections in vitro and transgenic mice in vivo, have suggested a role of oncogenically mutated c-erbB2 in the onset of breast cancer, which is supported by the observations that incidence of c-erbB2 overexpression (mainly due to gene amplification) in ductal cancer in situ is higher than that in infiltrating ductal carcinoma (Dickson et al., 1995). It was also reported that the EGFR and c-erbB2 seem to interact, through the formation of an interactive receptor species of high affinity that cross phosphorylates itself (Quian et al., 1992). Although c-erbB and c-erbB2 are closely related growth factor receptors, their relation to mammary lesions is somewhat different, as c-erbB is observed to be associated with the production of ductal lesions, whereas c-erbB2 (neu) is associated with alveolar lesions (Edwards et al., 1996).

The proto-oncogenes c-fos, c-myc, c-jun, and c-myb are commonly observed to be induced shortly after mitogenic stimulation of cells, and c-fos, c-jun, c-myc appear to be induced by estrogens and progestins in breast cancer (Van Der et al., 1991; LeRoy et al., 1991). The protein products of c-fos, c-jun are reported to function as transcription factor
through interaction with a target gene's promoter consensus sequence AP-1 (activator protein-1) site, while \( c-myc \) modulate gene expression through a different consensus sequence (Strange et al., 1995; Dubik et al., 1996). Amplification of \( c-myc \) gene, which seems to occur in all stages of breast cancer (Moffett et al., 1992), is one of the most common alterations in this disease, and it appears to be associated with postmenopausal disease and poor prognosis (Dubik et al., 1996). It has been proposed that Myc (the protein product of \( c-myc \)) is necessary for estrogen induction of proliferation of breast cancer cells (Watson et al., 1991; Dubik et al., 1996). It is also reported to be associated with TGF-\( \alpha \) overexpression and clonal expansion (Hall et al., 1990; Edwards et al., 1996). In the transgenic mice model system, TGF-\( \alpha \) and \( c-myc \) were shown to be strongly synergetic in induction of mammary tumors (Dickson et al., 1995). In addition, \( c-myc \) amplification was observed to enhance mammary and other cell types responses to growth-promoting effects of EGF and FGF (Valverius et al., 1990). It was also reported that Myc protein bind and inactivate the retinoblastoma tumor suppressor gene product (Rusty et al., 1991).

It has been postulated that cellular transformation from normal to abnormal phenotype may result from the effects of growth factors such as increased production of growth-stimulatory factors or decreased production of growth-inhibitory factors; or alternatively the transformation may be due to altered responsiveness to either or both groups of growth factors (Minden et al., 1992). In normal breast tissue, local growth factors functions may include stimulation (e.g. by members of TGF-\( \alpha \), FGF, EGF families), or inhibition (e.g. by TGF-\( \beta \) and other inhibitory factors) of epithelial proliferation (Dickson et al., 1995). EGF, a growth factor present in milk, appears to play an important regulatory
role in both proliferation and differentiation of mammary gland, and may be critical for the mammary carcinogenic process (Snedeker et al., 1991; Dickson et al., 1995).

Several studies have implicated the transforming growth factor families (TGF-α and TGF-β) in breast cancer, both of which are under transcriptional control by estrogens (and antiestrogens), however the effects of progestins (and antiprogestins) are not well clarified (Dickson et al., 1995). For example estrogen treatment of breast cancer cell lines under anchorage-dependent conditions was reported to stimulate proliferation, partially through induction of TGF-α and inhibition of TGF-β (Kenney et al., 1993), while progestin, partially via TGF-β induction, inhibited proliferation of cancer cells (Colletta et al., 1991). In contrast, under anchorage-independent conditions, both estrogen and progestin are growth stimulatory, partially through TGF-α induction and TGF-β inhibition (Ahmed et al., 1991).

Members of the TGF-α family are generally growth stimulatory, and studies of human breast cancer biopsies have shown that TGF-α mRNA and proteins were detected in more than 70% of the specimens, and approximately 30% of benign breast lesions (Gregory et al., 1989). Although studies of TGF-α expression in MMTV-induced mammary cancer in nude mice have suggested that TGF-α expression may be important in normal gland development and early stages of mammary carcinogenesis (Jhappan et al., 1990; Dickson et al., 1995), other studies have implicated TGF-α in cellular morphologic and malignant transformation (Sandgren et al., 1990; Matsui et al., 1990; Ciardiello et al., 1990), and the co-transfection of TGFα and c-erbB2 was shown to be capable of additive action to promote anchorage-independent growth (Ciardiello et al., 1992). The TGF-β family consists of at least three related gene products, all of which were detected in breast cancer, and their
interaction with TGF-β receptors appears to be complex. (Massague et al., 1992). Treatment of both normal and malignant epithelial tissues with subtypes of TGF-β had, in general, a growth-inhibitory and sometimes differentiating effects (Dickson et al., 1995). However, some studies in vivo with breast cancer implants in nude mice have suggested that TGF-β may stimulate tumor growth and suppress immune functions (Zugmaier et al., 1991). Also contradictory effects of TGF-β regarding angiogenesis have been reported, where TGF-β inhibited endothelial proliferation in vitro, but a focal injection in vivo stimulated angiogenesis (Folkman, 1996). Other inhibitory factors that may play a role in breast cancer include MDGI, mammostatin, and α-lactalbumin (Dickson et al., 1995).

Other growth factors (and their binding tyrosine-kinase receptors) which are associated with breast cancer include IGF-II, HGF, PDGF, MDGF-1, and FGFs. While IGF-I is reported to be expressed by normal breast fibroblasts, IGF-II is expressed by breast tumor-derived fibroblasts. The HGF, implicated in regulation of both cell growth and motility, has been shown to bind and activate as a ligand the c-met (a tyrosine-kinase receptor) (Dickson et al., 1995). The MDGF-1, found in human milk, was shown to stimulate the stromal collagen production, and may also play a role in growth regulation of normal and malignant human mammary epithelium (Bano et al., 1992). The transfection of FGF-4 in hormone-independent breast cancer cell lines was shown to enhance tumor growth and metastases (McLeskey et al., 1993). Several studies have examined the relationships of estrogens and progestins with several growth factors' gene products as mediators and modulators of steroid hormone actions, and several gene products were shown to be differentially regulated by estrogen and progesterone, however, the study of these interactions in human mammary
tissue has often been limited to malignant epithelium (Musgrove et al., 1991; Krusekopf et al., 1991; Buta et al., 1992; Kenney et al., 1993; Dickson et al., 1995).

In contrast, the tumor suppressor genes (which inhibit cellular proliferation in their normal configuration and are also implicated in the induction of apoptosis) contribute to the carcinogenic process when they are inactivated, thus depriving the cell of their growth inhibitory effects (Fornace et al., 1992; Squire et al., 1992; Levine et al., 1995). The loss of heterozygosity (LOH), an indicator of altered suppressor gene, appears to be common at multiple chromosomal sites suggesting that multiple tumor suppressor genes may be altered in cancer, or inversely that the collective presence of normal suppressor genes may be required to suppress the disease (Sato et al., 1991; Muncaster et al., 1992). Tumor suppressor genes can generally be classified as: {1} Genes that code for growth inhibitory products or their receptors such as genes for TGF-β and its receptors. {2} Genes that code for cytoplasmic relays (second messengers) in inhibitory signaling pathways such as APC; DPC4 (code for rely molecules in signaling pathway that inhibits cell division), NF-1 (codes for a protein that inhibits the growth stimulatory p21 protein), and NF-2. {3} Genes that code for proteins in the nucleus such as p15 (codes for a protein which in normal response to signaling from TGF-β inhibit cell cycle progression, and is involved in a variety of cancers), MTS1 (codes for the p16 protein, an inhibitory component of the cell cycle clock and is involved in a wide range of cancers including breast cancer), RB (retinoblastoma, which codes for the RB protein, a master inhibitory component of cell cycle and is involved in a wide variety of cancers, including breast cancer), p53 (codes for the p53 protein, a major inhibitor of cell cycle, and is involved in a wide range of cancers, including breast cancer),
and WT1. The p53 gene is the most studied tumor suppressor gene, and in humans the p53 product is reported to act as transcriptional activator which plays important roles in cell cycle checkpoint control, gene amplification, DNA repair, and apoptosis among other cellular functions. (4) Genes that code for other types of growth inhibitory molecules such as BRCA 1 (which is involved in breast and ovarian cancers), BRCA 2 (involved in breast cancer), and VHL gene. BRCA-1 and BRCA-2 seem to occur in the majority of familial breast cancers, as well as a substantial proportion of familial ovarian cancer (Kuerbitz et al., 1992; Fornace et al., 1992; Kastan et al., 1992; Yin et al., 1992; El-Deiry et al., 1993; Lu et al., 1993; Levine et al., 1995; Dickson et al., 1995; Weinberg, 1996).

Of the well-characterized alterations observed in human breast cancer are the loss or mutation of the tumor suppressor genes p53, RB, BRCA-1 and -2, nm23, and CDK4 inhibitor, particularly RB and p53 genes (Malkin et al., 1990; Marx, 1990; Thorlacins et al., 1991; Dickson et al., 1995). It has been estimated that about 10% of all breast cancers (10-15% of premenopausal cancers) show familial pattern, and in those cancers, the BRCA-1 gene locus is consistently deleted or rearrangement (Lancaster et al., 1997). The loss of the tumor suppressor gene nm23 (whose normal product seems to have the ability to attenuate metastasis in breast cancer model systems) appears to parallel the metastatic progression of breast cancer (Dickson et al., 1995). The altered genes may also interact. For example, BRCA-1 alterations appear often to be associated with amplification of c-erbB, in breast tumors (Sato et al., 1991), and both RB and p53 seem to be lost with high frequency as breast tumor progresses through various stages of malignancy (Davidoff et al., 1991; Osborne et al., 1991). The loss of expression of protein tyrosine phosphatases is also thought to enhance
the growth promoting and oncogenic activities of tyrosine-kinases such as EGFR and c-erbB2 (Saito et al., 1991; Fisher et al., 1991; Zhai et al., 1996).

**Cell Cycle.** The interval between each cell division, defined as a cell cycle, differs in length from tissue to another, however, it is characteristically short in tumor cells. Progression through the cell cycle is *driven by* the rising levels of the different cyclins and the cyclin-dependent Kinases (CDKs); is *controlled by* the RB protein and other inhibitory proteins such as p21 (under control of p53), p15 protein (induced in response to signals from TGF-β), p16 and p27, all of which block the activity of CDKs; and is *governed by* the crucial check points control (Murakami et al., 1995; Weinberg, 1996). There is also evidence that the nuclear chromatin structure regulates gene expression and cell cycle progression (Lee DY et al., 1993). The best characterized cdk-inhibitory protein is the p21WAF, believed to exert its growth inhibitory effects largely during G1 phase but was also shown to participate in the G2 Checkpoint that follow DNA damage (Gorospe et al., 1996), and its transcription is reported to be regulated by p53 as well as via p53-independent mechanisms (Macleod et al., 1995). It has been reported that breast cancer cells often produce excessive amount of cyclins such as cyclin D and E (commonly amplified in breast cancers), and they lack functional p53 proteins; whereas the cyclin-dependent kinase inhibitor-4 (CDKI-4) was reported to be commonly deleted or mutated in breast cancers (Keyomarsi et al., 1993; Kamb et al., 1994; Weinberg, 1996).

**DNA repair.** The inheritance of defects in DNA repair genes in humans is observed to be associated with cancer predisposition. People born with defective ATM gene for example were reported to be more susceptible to a variety of cancers, including breast cancer, and it
is proposed that as many as 10% of inherited breast cancers may occur in individuals with defects in this gene (Weinberg, 1996).

**Apoptosis.** Since normal development and maintenance of tissue size depend on a balance between cell division and programmed cell death (apoptosis), perturbation of this balance can contribute to the development of neoplasia (Allan et al., 1992; Schedin et al., 1996; Russo et al., 2001). Apoptosis is an important mechanism to minimize the consequences of allowing the survival and propagation of damaged cells, where prolonged survival can permit the further accumulation of alterations associated with neoplastic transformation (Andres et al., 1991). The apoptotic pathway can be activated by treatment with physical or chemical agents, the activation of an oncogene or the inactivation of a tumor suppressor gene within the cell. For example, functional p53 is implicated in activating a pathway leading to apoptosis, whereas cells that expressed altered forms of p53 were observed to exhibit marked resistance to the lethal effects of radiation (Eastman et al., 1990; Yonish et al., 1993; Lee JM et al., 1993). Also cancer cells were observed to often overproduce the Bcl-2 protein, reported to efficiently inhibit apoptosis (Weinberg, 1996).

**Angiogenesis.** Recent research have supported the earlier hypothesis that tumor growth is angiogenesis-dependent, and have demonstrated the importance of neovascularization (angiogenesis) to the invasiveness and metastatic dissemination of cancer. It was reported that the degree of metastasis is directly proportional to the number of capillaries infiltrating the tumor (Weidner et al., 1991; Weidner, 1996; Folkman, 1995). The regulation of the angiogenic process has not yet been fully elucidated, and the reasons for some tumors such as in the cervix to undergo neovascularization much earlier than others are not yet known.
However, it was proposed that a significant component of the angiogenic process is due to endothelial-cell migration, capillary budding, establishment of capillary loops, and/or neovascular modeling (Weidner, 1996). It was reported that release of heparin-binding growth factors such as bFGF is closely associated with angiogenesis (Kandel et al., 1991; Wellstein et al., 1992), and the expression (transfection) of the FGF-4 (hst) gene was associated with promoting lymphatic/hematogenous metastasis (Murakami et al., 1990; Dickson et al., 1995). The amplification of FGF-3 (int-2) gene and FGF-4 (hst) gene (both located at 11q13 chromosome) was observed to occur in human breast cancer, and that amplicon also reported to include the cyclin-D1 gene (Dickson et al., 1995).

It is worthy to note that, in certain cases, the switch to the angiogenic phenotype appears to occur independently of other activities that arise during tumorigenesis, and may occur before full transformation to malignant stage, i.e. in the preneoplastic or the preinvasive stages. (Weidner et al., 1991; Smith et al., 1994; Folkman, 1995; Weidner, 1996). This insidious property of the angiogenic process, greatly complicate the prospects of early detection and prognosis, because by the time a breast cancer lesion could be seen on a mammogram, the tumor may have already undergone neovascularization (Folkman, 1995), which increases the probability of being already metastasized. In turn, the newly proliferated endothelial cells may release different factors that can stimulate the proliferation and/or motility of tumor cells. For example, it was reported in breast cancer that the capillary endothelial cells recruited to the tumor site produce a cytokine (IL-6) which may increase the potential of cancer cells migration into the blood stream and thus metastasis (Motro et al., 1990; Folkman, 1996).
Cancer (and inflammatory) cells may induce different naturally occurring vascular growth factors that promote new capillaries formation (Weidner, 1996). The reported positive regulator of angiogenesis include VEGF, PDEGF, FGF, TGF-α, TNF-α, IL-8, and HGF (Folkman, 1995). Several non peptide molecules have also been identified to be angiogenic including prostaglandin E₁ and E₂, nicotinamide, adenosine, and certain metabolites of hyaluronic acid (Folkman, 1995), and selective COX-2 inhibitors were shown to exhibit antiangiogenic and anti-tumor activities (Masferrer et al., 2000). On the other hand, several natural negative regulator of angiogenesis have been reported such as PF4, thrombospodin (which may be normally under the control of the p53 tumor suppressor gene), TIMPs, INF-α and -β (Fidler et al., 1993; Pientenpol et al., 1993; Folkman, 1994; 1996; Weidner, 1996).

In animal models, it was shown that breast cancer tissue implanted in mice and allowed to grow, can be reduced to a microscopic size and held in a dormant state for as long as an antiangiogenic agent (angiostatin) is administered (O’ Reilly et al., 1996). Also, studies in animal models have shown that combination of antiangiogenic agents and chemotherapeutic agents are more effective than either therapy alone, and antiangiogenic agents increased the effectiveness of radiation therapy (Teicher et al., 1995). Therefore, after chemotherapy or radiation therapy, angiogenesis inhibitors might be of use as a long term therapy against cancer, particularly with metastatic lesions.

**Immortality.** Another mechanism that contribute to carcinogenesis is immortality (as opposed to senescence and death). In contrast to apoptosis, the property of immortality may give the tumor cells the time to accumulate additional alterations that may increase their
ability to replicate, invade and metastasize. It was reported that cells with sustained inactivation of tumor suppressor genes $p53$ or $RB$ continued to divide and become immortal after their intact counterparts enter senescence, and these abnormal cells along with their progeny can multiply indefinitely (Weinberg, 1996). The integrity of the telomere, the molecular component that underlie the mechanism of monitoring the number of replicative generations and at certain point initiate senescence and cell death, is maintained in tumor cells through the induction of the telomerase activity, thus enable tumor cells to replicate indefinitely (Meeker et al., 1996; Weinberg, 1996). Since the majority of human cancers have telomerase activity while most normal cells do not, telomerase inhibition has been proposed as a target for anticancer therapy; and inhibition of tumor cells’ telomerase activity by various agents such as hormones, differentiating agents, and selective COX-2 inhibitors, was reported to have anti-tumor effects (Meeker et al., 1996; Nishimura et al., 1999).

**Malignant transformation and Metastasis.** Although several genes and signal pathways have been associated with the malignant and metastatic properties, the precise mechanisms that underlie these processes are not fully clarified. During the progression of breast cancer, two interactive processes seem to occur: {1} loss of differentiation properties (malignant transformation); {2} loss of proper tissue compartmentation (invasiveness and metastasis). The term malignant transformation is used here to denote the transformation processes where the premalignant cells acquire all the properties of fully transformed (malignant) cells such as heightened tumorigenic potential, anchorage independence, media requirements-independence, disorganization of actin filaments, secretion of plasminogen activator to produce fibrinolysis, loss of contact inhibition, and increased motility potential.
In case of malignant transformation, it appears that three molecular determinants, namely alterations in cell-cell attachment, cell-matrix attachment and cytoskeletal organization, are critical to this process. The normal cells' ability to recognize their place in the tissue depends on receptors and adhesion molecules, and two kinds of adhesion, cell-cell adhesion and adhesion to the extracellular matrix (ECM) seem to play critical roles during tissue invasion and metastasis (Ruoslahti et al., 1996). For example, the loss of expression of cell-cell adhesion molecule, E-cadherin, in breast cancer cells was associated with differentiation degradation, in the form of acquiring more fibroblastic morphology and the expression of vimentin, an intermediate filament of the mesenchymal cells, as well as increased motility and invasiveness of the basement membrane (Sommers et al., 1991; Thompson et al., 1992), and the loss of E-cadherin has been shown to turn a cultured lineage of cells from non-invasive to invasive phenotype (Akiyama et al., 1996). This differentiation-degrading process, termed EMT (epithelial-mesenchymal transition) - which is observed to occur frequently during embryogenesis (Navarro et al., 1991) - was reported to be associated with primary defects in the arrangement of desmosomal and cytoskeletal proteins (Ciardiello et al., 1992). However, the exact mechanism of EMT remain to be clarified. The loss of ER and PR expressions was also associated with the EMT process in breast cancer (Cohen et al., 1991). Cell-matrix attachment also appears to be critical in differentiation and in metastasis (Liotta et al., 1991; Streuli et al., 1998). The loss of the \( \alpha_\beta_1 \) integrin attachment molecule (the fibronectin receptor) was implicated in breast cancer progression (Dickson et al., 1995). Anchorage dependence or adhesion to extracellular matrix, mediated by cell-surface molecules such as the integrins including the cell receptors
for fibronectin (an ECM protein), was observed to be lost in cancer cells (Bernstein et al., 1994), and in anchorage-independent cancer cells, the cyclin/CDK complex (involved in cell division) was observed to remain active whether the cells are attached or not (Ruoslahti et al., 1996). The expression of high levels of the laminin receptor (a non integrin receptor, strongly induced by estrogen and progesterone in breast cancer cell lines) has also been observed to correlate with progression of breast cancer (Liotta et al., 1991). Another matrix component, tenascin, appeared to be synthesized in areas of local invasion as well as invasion into metastatic sites (Dickson et al., 1995).

In addition to alterations in cell-cell and cell-matrix attachment, and cytoskeletal organization, the cell locomotion, proteolysis, and the ability to survive and proliferate at distant sites seems to influence the processes of local invasion and metastasis (Rusciano et al., 1992). Cancer cells (like white blood cells) can penetrate basement membranes and invade extracellular matrices through the release of enzymes such as the various metalloproteinases (which include gelatinases, the isoforms of type IV collagenase, and stromelysins); plasmin (caused by the tumor cell secreted plasminogen activator, PA); Cathepsins (lysosomal matrix-degrading cysteine proteinases, known to be under hormonal control), and the glycosidases (Groendahl et al., 1993; Ruoslahti et al., 1996; Kantor et al., 1996; Ren et al., 1996). The mammary stromal production of the protease stromelysin-III and other stromelysins were implicated in the early invasion of breast cancer (Lamacher et al., 1990; Basset et al., 1996). Proteolysis may depend on the balance between the enzymes and their inhibitors, for example, the MP-1 and -2 are secreted with endogenous tissue inhibitors of metalloproteinases called TIMP-1 and -2 (Dickson et al., 1995).
The invasiveness of breast cancer is also marked by abnormal stromal-epithelial interactions. The stimulation of cell motility is a complex process involving the production of motility factors (by both the mammary epithelium and stromal cells), and the recognition of motility factors by membrane receptors, where cells may respond both directionally (chemotaxis) and randomly (chemokinesis) to stimulation by such soluble factors (Kantor, 1996). Reported factors which may act locally to increase cell motility include AMF, the fibroblasts' cytokine HGF/SF (which acts through the c-myc tyrosine kinase oncogene), bFGF, VEGF, and PD-ECGF. In addition, systemic cytokines such as MSF, and the cytokine IL-6 affect the motility of tumor and/or non tumor cells (Kantor et al., 1996). PKC was also implicated in promoting cellular motility, since PKC expression seems to increase during malignant progression, and because a primary substrate of PKC is an actin filament cross-linking protein thought to be involved in motility (Isakov et al., 1991; Hartwig et al., 1992).

Hormonal influences are also of importance in growth control of metastatic breast cancer, as (ER-) mammary cells were reported to be more invasive than (ER+) cells (Thompson et al., 1992), and estrogen was shown to enhance the invasiveness of (ER+) cell lines (Dickson et al., 1995). Interestingly, tamoxifen was reported to be sufficiently estrogenic to induce invasiveness of breast cancer cell lines, in contrast to a pure antiestrogenic agent which was shown to be suppressive (Dickson et al., 1995). Certain dietary fats were also reported to strongly modulate tumor growth and metastatic spread in experimental models of breast cancer (Noguchi et al., 1992; Karmali et al., 1993; Rose et al., 1997).

The presence of motility factors in and around the tumor cells, however, appears to be necessary but not sufficient to produce metastasis. As tumor cells progressed from
premalignant to malignant lesions more changes in gene expressions appear to occur, and such metastasis associated genes include Mts-1, the subtype nm23-H1 of the nm23 gene, and c-erbB, (Dickson et al., 1995; Kantor et al., 1996). It is estimated that probably fewer than one in 10,000 cancer cells that reach the circulation survives to establish a new colony at a distant site (Ruoslahti et al., 1996), but the reason for this vulnerability in the bloodstream are not well understood. Surviving cancer cells' lodging to a new site may be enhanced by the aggregation of blood platelets, which are also known to produce growth factors, and in some experimental systems, drugs that interfere with platelet functions, such as NSAIDs, have been shown to have anticancer effects (Ruoslahti et al., 1996).

1.3.4. Premalignancy in mammary tumorigenesis.

The multistage carcinogenic model implies that cancer in epithelial organs may proceed via multiple intermediate stages. The intermediate premalignant stage in mammary gland have been labeled with different terms, and a well descriptive term being proliferative breast disease (Miller, 1996). Several experimental model systems in rodents, using mouse mammary tumor virus (MMTV), chemical carcinogens, prolonged hormonal stimulation, oncogenes, and growth factors, have been developed that describe the premalignant phenotypes of mammary tissue (Medina, 1996). These phenotypes were generally characterized as stably hyperplastic in morphology, immortalized, and dependent on local factors that regulate growth and spacing in mammary fat pad (Medina, 1996). Many of the premalignant lesions, which are not terminally differentiated lesions, are reported to be ovarian hormones-independent for growth or morphologic differentiation, yet retain hormone responsiveness for functional differentiation (Medina, 1996). Cumulative data from these
experimental studies on the possible roles of oncogenes, tumor suppressor genes, cyclines and growth factors in the development of mammary hyperplasia have implicated ras, erbB-3, erbB-4, p53, cycline D1, D2, E2, p34 tyrosine phosphorylation, TGF-α, EGF, PDGF, MDGFI, TGF-β, and IGF-I and-II (Medina, 1996). In human preneoplastic cell-line models, overexpression of ras and nm23 (metastasis-related gene) and decreased expression of p53 were associated with increasing malignancy (Miller, 1996).

It is worthy to note that, due to the presence of a number of histological and biological differences between animals and humans mammary lesions, the absence of a well defined model of breast cancer development, as well as the imprecise characterization of the fibrocystic complex, the notion of whether a premalignant lesions exist in the development of human breast cancer is still a point of contention. It is not fully clear whether the lesions considered premalignant in human breast are precursors of malignant lesions or markers for high risk (Page et al., 1992). Although atypical hyperplasia (the example often referred to as human hyperplastic premalignant lesion) is observed in human breast and is associated with increased risk of cancer, its exact role in tumor progression is not fully understood (Miller, 1996).

1.3.5. The DMBA-induced Animal Mammary Cancer Model:

Two rat models are preferentially utilized in the study of mammary carcinogenesis, the female Sprague Dawley (SD) rat inoculated with DMBA, and SD or Fischer 344 rat injected with NMU(nitroso methyl urea). Studies of mammary development in rats have revealed a resemblance to human breast development. For instance, at the time of pubertal mammary growth in the rat (about 25-35 days of age), the active growth centers, the terminal

50
end buds (TEBs), start to bifurcate into alveolar buds (Abs) which progress to virginal lobules with successive estrous cycles (Russo & Russo, II, 1997). Several interesting insights are provided by the chemically-induced rat mammary carcinogenesis model. It provides evidence that chemical carcinogens are causative agents of mammary cancer; that the initiation of the disease requires the interaction of the carcinogen with undifferentiated and highly proliferative mammary gland tissue; and that full term pregnancy can protect the gland from tumorigenesis through the induction of differentiation (Russo & Russo, I, 1997). All are findings that are relevant to human breast cancer. The rat mammary gland is a known target tissue for polycyclic aromatic hydrocarbons (PAHs)-initiated carcinogenesis, and among the PAHs, DMBA (7,12-dimethyl benz[a]anthracene), was observed to be the most potent in inducing mammary carcinomas in young virgin Sprague Dawley rats (Christou et al., 1987). DMBA given by gavage in a single dose of 2.5 - 20 mg was reported to induce tumor incidence with latency periods that range generally between 8-21 weeks, with final tumor incidence almost 100% (Russo & Russo, II, 1997). The administration of DMBA to virgin rats has elicited a tumorigenic response whose incidence is directly proportional to the density of TEBs that are primed by the ovarian hormones to be differentiated to Abs (Russo & Russo, II, 1997). DMBA is metabolized by the rat mammary epithelium into both polar and phenolic metabolites, however, the formation of polar metabolites (which form the DNA adducts) was observed to be higher in epithelial cells of TEBs than the more differentiated lobules. On the other hand, removal of adducts from DNA was observed to be very low in the TEBs, whereas the lobules are more efficient (Russo & Russo, II, 1997). Although it has been observed that the administration of DMBA to rats between the ages of 30-55 days
produce 100% tumor incidence, the highest number of tumors/animal, i.e. tumor burden (multiplicity), was observed to develop when the carcinogen is given to animals between the ages of 40-46 days, a period when TEBs are most actively proliferating and differentiating into Abs. Lower tumor incidence as well as tumor burden (multiplicity) were also observed to accompany the sharp decrease in the number of TEBs in animals older than 55 days of age (Russo & Russo, II, 1997). The fully developed or palpable tumors may grow into invasive carcinomas, and some tumors were observed to metastasize, mainly to the lung, if the animals are allowed to live long enough. DMBA was reported to induce benign lesions as well, which were observed to originate from more differentiated structures such as Abs and virginal lobules, however they were observed to appear later than the cancerous lesions, suggesting that there are two different pathogenic pathways, one for the malignant and another for the benign, and the benign lesions may not be the precursors for the malignant lesions (Russo & Russo, II, 1997).

1.4 Prostaglandin-H Synthases (COXs) and Prostaglandins.

1.4.1. PGHS-1 (COX-1) and PGHS-2 (COX-2):

To date, two isoforms of prostaglandin H synthase (PGHS) enzyme - also known as prostaglandin endoperoxide H synthase, prostaglandin endoperoxide G / H synthase, and COX (for cyclooxygenase) - generally referred to as COX-1 and COX-2 isoforms have been identified and both catalyze the formation of PGH₂ from arachidonic acid, through the cyclooxygenase and peroxidase reactions (Xie et al., 1991; Kujubu et al., 1991). PGHS-1 (COX-1) is referred to as the constitutive isoform, as opposed to PGHS-2 (COX-2), which
is referred to as the inducible isoform (Smith & DeWitt, 1996). It is worthy to note that the general classification of COXs as constitutive vs. inducible isoyme is actually an over simplification, since COX-1 gene is inducible in some contexts (Herschman et al., 1997), as levels change during development (Brannon et al., 1993), its expression can be up-regulated (Samet et al., 1995; DuBois et al., 1998) or down regulated (Hla et al., 1991), and was shown to be overexpressed in animals and human breast cancer (Hwang et al., 1998). However, it is in general not nearly as responsive to environmental stimuli as is COX-2 (Herschman et al., 1997). On the other hand, COX-2 is expressed constitutively in specialized cell types or tissues where it plays specific functions in individual biological processes (Vane et al., 1998). Therefore, characterization as constitutive or inducible, based on the extent / degree of expression, would be more appropriate.

**Structure & Catalytic Activity of COX Isozymes:** Although they may differ with respect to pattern of expression, COX-1 and COX-2 appear generally to be similar in structure, and they have similar gross kinetic properties. Both isozymes' cyclooxygenase reactive site is inhibited by general NSAIDs, although relatively specific inhibitors for each isozyme have been developed (Smith & DeWitt, 1996). Structurally, both isoforms are homodimeric, heme-containing, N-glycosylated proteins with two catalytic active sites. Each monomer is made up of three domains including a small N-terminal domain called EGF domain; a membrane-binding domain (MBD) which have four (A, B, C, D) short helices; and a large globular C-terminal catalytic domain (Smith & Garavito, 1996; Smith et al., 2000). Rather than anchoring to the lipid bilayer through trans-membrane motif typical of many integral membrane proteins, COXs are anchored only to one leaflet of the lipid bilayer by what is
proposed to be an interdigitation of side chains of hydrophobic amino acid residues emanating from the surfaces of these helices (Picot & Garavito, 1994; Smith & Garavito, 1996). It has been observed that COXs isozymes do function as dimers in the cellular membranes (Smith & DeWitt, 1996; Smith et al., 2000). There are three N-linked, high-mannose oligosaccharides chains attached to COX-1 and four chains attached to COX-2, which are considered essential for the isozymes folding and activity (Otto et al., 1993; Otto & Smith, 1994). Another unique difference between COX-1 and -2 is the presence of 18 amino acids inset near the C-terminus of COX-2 that is not present in COX-1 (Subbaramaiah et al., 1997). Although the function of this cassette is not fully established, it is thought to mark COX-2 for rapid proteolysis (degradation), or it may provide a signal for subcellular trafficking (Smith & DeWitt, 1996), and the elimination of this cassette was reported to have no apparent effects on COX-2 catalysis (Smith et al., 2000). Anti peptide monoclonal antibodies raised against this unique region, have been used to distinguish COX-2 from COX-1 (Habib et al., 1993; Smith & DeWitt, 1996; Subbaramaiah et al., 1997).

The crystal structures of the two isozymes were shown to be essentially superimposed (Smith & Garavito, 1996), and the primary sequences of COX-1 and -2 were reported to be 60% identical within a species, where the sequence differences between the two isoforms appear to occur primarily in the membrane binding domain (Subbaramaiah, 1997; Smith et al., 2000). As to the protein size, COX-1 migrate as a single band on SDS-PAGE with an apparent molecular mass of 72 kDa, and calculated (from amino acids sequence ) molecular mass of 66kDa; whereas COX-2 migrate as two bands on SDS-PAGE with apparent molecular mass of 72 and 74 kDa and calculated molecular mass of 67 kDa (Lecomte et al.,
1994; Subbaramaiah et al., 1997). The difference between the observed and the predicted values is considered a consequence of post translational addition of the N-linked oligosaccharide chains (Otto et al., 1993; Smith & DeWitt, 1996; Subbaramaiah et al., 1997).

The mature processed forms of COX-1 all have 576 amino acids, and the mature forms of COX-2 have 587 amino acids, and the first 3 amino acids of COX-1 are encoded by an exon of the COX-1 gene that is absent from the COX-2 gene (Smith et al., 2000).

Both COXs catalyze the conversion of arachidonic acid and O₂ to PGH₂, the committed step in the prostanoid biosynthesis pathway. Both COX-1 and COX-2 catalyze two reactions at two biochemically (functionally) and structurally distinct sites termed the cyclooxygenase (COX) and peroxidase (POX) active sites, respectively. Although the two active sites are separate, the heme prosthetic group is absolutely required for both activities (Ogino et al., 1978, Smith et al., 2000). While the mechanisms underlying activation of this prosthetic group are not fully known, it is thought to be through the binding the isozyme’s heme group ferrous (Salvemini et al., 1994), and replacement of the heme group with mangano-heme (Mg²⁺) produced COX with 5% and 40% of the respective peroxidase and cyclooxygenase activity of the native enzyme (Smith & DeWitt, 1996). The X-ray analyses have indicated that it is interposed between the isozyme two active sites (Picot et al., 1994; Luong et al., 1996; kurumbail et al., 1996).

In case of COX-1, the cyclooxygenase active site is a channel that is lined with hydrophobic residues, and certain key active site residues are reported to be involved in catalysis and governing the stereospecificity of COXs toward at least certain NSAIDs such as ibuprofen and flurbiprofen (Bhattacharyya et al., 1996). The gross structure of COX-2
cyclooxygenase active site is reported to be similar, only that it is 25% larger (partly due to the presence of a second internal pocket), and has a slightly different shape (Picot, 1994; Taketo, 1998; Smith et al., 2000). Three amino acid residues lining the channel were observed not to be identical between COX-1 and -2, and the position of helix D (one of the MBD helices) is different between COX-1 and -2 (Smith et al., 2000). The differences between the two isozymes active site’s structure were reported to have consequences in terms of more substrate flexibility for COX-2 (effectively oxygenates a wider array of fatty acid substrates than COX-1), and lower relative affinities of NSAIDs for COX-2 (Taketo, 1998). Also the difference in size and shape of the NSAIDs’ binding site are reflected in catalytic difference between the isozymes. For example, in acetylation of COX-1 by aspirin, the acetyl group protrudes into the cyclooxygenase active site and interfere with arachidonate binding, and this covalent modification of COX-1 by aspirin produces permanent inactivation of the enzyme (Loi et al., 1995). In contrast, aspirin-acetylated form of COX-2 retains cyclooxygenase activity, but produce a certain product, 15R-HETE (Taketo, 1998). The structural differences have also been exploited in developing selective inhibitors of COX-2 (Picot et al., 1994; Luong et al., 1996; kurumbail et al., 1996; Taketo, 1998). Compounds that were designed to bind to the additional space and different residues are potent and selective inhibitors of COX-2 (Subbaramaiah et al., 1997).

The gross kinetic properties (e.g. $K_m$, $V_{max}$) of the two isoforms are similar in the overall context. For example, the cyclooxygenase turnover numbers (approximately 3500 mol. of arachidonate/ min/ mol. of dimer), and the apparent $K_m$ values for arachidonate (approximately 5 uM) and $O_2$ (approximately 5 uM) are about the same for the two isozymes
(Laneuville et al., 1995; Smith & Garavito, 1996). However, kinetic differences between COX isozymes were demonstrated, where COX-1, but not COX-2, was reported to exhibit negative allosterism at low arachidonate concentration, which may permit COX-2 to compete more effectively for newly released arachidonate when both isozymes are expressed in the same cell (Swinney et al., 1997; Kulmacs et al., 1998; Smith et al., 2000). In addition, COX-2 has a significantly lower threshold for hydroperoxide activation than COX-1, thereby enabling COX-2 to oxygenate arachidonate in the presence of lower peroxide concentrations (Spencer et al., 1998).

Both COX-1 and -2 have very active peroxidase catalytic site located near the surface of the protein on the opposite side of the heme group from the cyclooxygenase active site (Picot et al., 1994). It was shown *in vitro* that the peroxidase activity of both COXs can reduce a variety of peroxides such as \( \text{H}_2\text{O}_2 \) and 15-HPETE, however, both isozymes showed a preference toward secondary alkyl hydroperoxides such as the physiologically important substrate \( \text{PGG}_2 \), but it is not clear which amino acid residues govern such specificities (Smith & DeWitt, 1996; Smith et al., 2000). Both the gross structure of COXs peroxidase active sites, and the mechanisms of peroxidase catalysis seem to be similar to those of other heme peroxidases (Smith & DeWitt, 1996). The peroxidase reaction basically involves two electrons oxidation of the heme group to form oxyferryl intermediate that is reconverted back to the native (resting) ferric heme by two electrons reduction (Smith & Marnett, 1994; Picot et al., 1994; Smith & DeWitt, 1996; Swinney et al., 1997; Smith et al., 2000). *In vitro*, hydroperoxide preparations were shown to oxidize the heme groups of a fraction of COX molecules, and the remaining molecules are then automatically activated by the newly
generated PGG₂ (Smith et al., 2000). The identity of the agent(s) that initiate heme oxidation 
*in vivo* is not yet known, but peroxynitrite (that may be derived from the condensation of 
nitric oxide and a superoxide) may serve as a physiological heme oxidant (Smith & DeWitt, 
1996; Smith et al., 2000; Marnett et al., 1999). Interestingly, the factors that modulate the 
expression of the inducible forms of nitric oxide synthase (iNOS) also seem to regulate the 
expression of COX-2 (Marletta et al., 1994; Mei et al., 2000). In addition, nitric oxide has 
been reported to activate both COXs *in vitro*, and in animal models *in vivo* (Salvemini et al., 
1993; 1994; Goodwin et al., 1999). The second component of the peroxidase cycle is the 
reduction of the intermediate compound’s heme group by the substrate present (fatty acid or 
otherwise) and the reducing co-substrates, which provide reduction electrons (Swinney, 
1997; Smith, 2000). The possible *in vivo* reducing co-substrate that can reduce the heme 
group include glutathione, ascorbate, epinephrine, and uric acid (Smith & DeWitt, 1996). 
There are some subtle differences reported for the peroxidase activity exhibited by COX 
isozymes. For example, the rate of formation of intermediate compounds is much faster with 
COX-2 (Smith et al., 2000); and at relatively high (50uM) hydroperoxide concentration 
COX-1 was reported to catalyze a standard two-electron reduction of the hydroperoxidase 
substrates almost exclusively, whereas COX-2 catalyzes 60% two-electron and 40% one-
electron reductions resulting in changed products profile (Landino et al., 1997).

There is an interdependence of cyclooxygenase and peroxidase reactions in that the 
cyclooxygenase reaction is peroxidase dependent, and this interplay of the two activities 
could be illustrated through a branched-chain mechanism (Kulmacs et al., 1994; Smith & 
DeWitt, 1996; Landino et al., 1997; Lu et al., 1999; Smith et al., 2000). However, the
peroxidase activity of COXs can function independently of the cyclooxygenase activity (Smith & DeWitt, 1996; Smith et al., 2000). It is noteworthy that the peroxidase activity of COXs can also catalyze the peroxidatic co-oxygenation of xenobiotics, such as synthetic estrogens, polycyclic hydrocarbons, aromatic amines, nitrofurans, mycotoxins, hydantions, bisulfite, phenols, heterocyclic amines, and indoles, leading to the production of intermediates that may undergo secondary chemical reactions depending on their solution chemistry. These processes have been suggested to be more important in xenobiotics metabolism in tissues having low levels of cytochrome P450 (Smith & DeWitt, 1996; Smith et al., 2000).

Deactivation (proteolysis) and suicide inactivation: The turnover of COX-2 is more rapid than that of COX-1, and it is proposed that the 18-amino acids' cassette of COX-2 may influence the rate of enzyme degradation (Smith & DeWitt, 1996; Smith et al., 2000). It has been reported that COX-1 and -2 isozymes can be cleaved by trypsin into two fragments at different sites. However, trypsin was observed not to cleave the enzyme in the presence of heme and/or NSAIDs, and it is not obvious why these agents protect the enzyme from trypsin cleavage (Smith & DeWitt, 1996).

It has been observed that addition of arachidonate to preparations of COXs would result in rapid but transient burst in O₂ consumption as PGH₂ is formed, then both the cyclooxygenase and peroxidase activity would fall to almost zero within 1-2 minutes even in the presence of sufficient substrates. On average every COX molecule was reported to consume approximately 400 arachidonate molecules before becoming inactivated, and the presence of peroxidase reducing co-substrates markedly slowed the rate of inactivation of
COX (Kulmacs et al., 1994; Smith et al., 2000). Not much details are known about the precise chemical changes that occur during the observed phenomenon of rapid fall in COX activity, known as suicide inactivation, however, it was shown not to be due to product inhibition, but may rather result from a mechanism-based inactivation of the enzyme, probably involving forming an inactive enzyme intermediate species during the reaction (Smith & Garavito, 1996; Smith et al., 2000). Both COXs appear to have similar rate of suicide inactivation (approximately 30 seconds on average) (Smith & DeWitt, 1996). The physiological significance of suicide inactivation is not yet determined, and in general, the amount of COXs in intact cells are in excess of substrates, and bursts of PGs production by cells were observed not to lead to major losses in COX activity (Smith et al., 2000).

Structure of COX Genes: It has been reported that COX-1 and COX-2 are encoded by two separate genes, located on two different chromosomes. The gene for human COX-1, on chromosome 9 (map to 9q32-q33.3), is approximately 22 kilo base (kb) pairs and contains 11 exons and 10 introns, and its mRNA size is 2.7 kb (Taketo, I., 1998). The COX-1 gene lacks a TATA box, a promotor element commonly lacking in constitutively expressed housekeeping genes and a typical characteristic of developmentally regulated genes (Kraemer et al., 1992; Silvia et al., 1994; Subbaramaiah et al., 1997). The first two exons of COX-1, which contain the translational start sites and signal peptides, are condensed to form a single exon in COX-2, but the remaining intron/exon arrangement of the two genes are similar, however, the size of the introns in both isozymes are very different, where the introns of COX-2 gene are considerably smaller than those of COX-1 (Smith & DeWitt, 1996; Subbaramaiah et al., 1997).
The human COX-2 gene, located on chromosome 1 (map to 1q25.2-q25.3), is 8.3 kilo base (kb) pairs in length, and contains 10 exons and 9 introns, with mRNA size of 4.5 kb that contains multiple Shaw-Kamen sequences (Kujubu et al., 1992; Kosaka et al., 1994; Subbaramaiah et al., 1997; Taketo, I, 1998). The mRNA of COX-2 is relatively unstable compared to that of COX-1, which has been attributed to the presence of multiple copies of the Shaw-Kamen (ATTTA) sequences (Kosaka et al., 1994; Sheng et al., 1998). The small size of the COX-2 gene, as well as the presence of the instability sequences are consistent with its characterization as an immediate-early gene (Smith & DeWitt, 1996; Subbaramaiah et al., 1997).

Several responsive regulatory elements involved in COX isozymes transcription have been identified. The COX-1 gene has a TATA-less promotor that contains multiple start sites for transcription (Xu et al., 1997). Two Sp1 (sequence specific protein-1) regulatory elements in human COX-1 promotor, which bind the ubiquitous transcription factors Sp1 proteins and contribute to the constitutive expression of COX-1 in humans, have so far been identified (Smith et al., 2000). On the other hand, the 5' flanking region of the human COX-2 promotor contains a TATA box (motif) and transcriptional control elements necessary for activation of COX-2 (Inoue et al., 1995; Smith & DeWitt, 1996; Smith et al., 2000). Although several relevant enhancer sequences have been identified in human COX-2 gene promotor, five regulatory elements were rigorously demonstrated to regulate transcription, including two NFkB sequences, NF-IL6, E-Box, and overlapping ATF-CRE sequences (Subbaramaiah et al., 1997; Smith et al., 2000). The most critical of these regulatory sequences is considered to be the ATF-CRE sites (Sirois et al., 1993), which are typically
activated by transcription factors such as the dimers of c-jun (AP-1), member of ATF family, as well as the cAMP response element binding protein (CREB) (Inoue et al., 1994; Xie et al., 1995; Xie & Herschman, 1996; Subbaramaiah et al., 1996; Smith et al., 2000). Studies of the effects of ATF family of transcription factors such as CREB, and c-Jun (AP-1) on COX-2 promotor activity have revealed c-Jun as a more potent inducer of COX-2 than CREB (Subbaramaiah et al., 1996). The AP-1 transcription factors can also modulate transcription via a CRE response element (Pedraza et al., 1994). The rat COX-2 gene, however, does not contain a consensus CRE sequences, suggesting that regulation of COX-2 gene in these species may be different (Sirois et al., 1993). The NF-IL6 regulatory element, frequently found in promoters of acute phase genes, was also shown to bind the C/EBP proteins (cAMP enhancer binding protein, a family of transcription factors activated by most of the inflammatory stimuli that induce COX-2 expression), which appear not to work independently but to cooperate with other transcription factors such as USF-1, NFkB and the c-Jun (AP-1) regulatory proteins in activating transcription from COX-2 promotor (Gallois et al., 1998; Smith et al., 2000). The NF-IL6 regulatory element was shown to mediate, in part, the increased COX-2 transcription in rat follicles' following exposure to follicular-stimulating, luteinizing, and gonadotropin releasing hormones (Sirois et al., 1993), and the E-box sequence is considered essential for their stimulated transcription of COX-2 (Morris et al., 1996). The E-box appears not to be involved, however, in regulation of COX-2 in human epithelial cells (Smith et al., 2000). In humans vascular endothelial cells, both ATF-CRE and NF-IL6 sites were shown to be cooperatively responsible for transcription of COX-2 by lipopolysaccharide (LPS) and tumor promoting phorbol ester (TPA) (Inoue et al., 1995),
whereas both NFκB and NF-IL6 sites are responsible for COX-2 transcription by the tumor necrosis factor-α (TNF-α) in MC3T3-E1 osteoblasts (Yamamoto et al., 1995). A number of other elements including SP1 and ETS, which appear to be involved in regulation of other inflammation-related proteins, were also identified in COX-2 promoter region (Muller et al., 1993; Smith & DeWitt, 1996).

Regulation of COX Isozymes Gene Expression. An important distinction between COX isozymes involve their differential expression. The range of expression of COX-1 is 2-4 folds, whereas COX-2 can increase from 10- to 80- folds (Subbaramaiah et al., 1997). It has been reported that COX-1 can be detected in most tissues, although not in all cells within the tissue, and is typically expressed in cultured cells at constant levels throughout the cell cycle, hence COX-1 is generally viewed as the constitutive isozyme (DeWitt et al., 1993; Smith & DeWitt, 1996). Recent studies have shown that COX-1 may behave as a delayed response gene (Gallois et al., 1998). The relatively high levels of preferential expression of this isozyme, in some highly differentiated and specialized tissues and cells such as macrophages, monocytes, platelets, endothelial cells, seminal vesicles, gastrointestinal epithelial cells, and renal collecting tubules, have suggested that COX-1 expression is predominantly developmentally regulated (Smith et al., 2000). In cell lines that undergo differentiation (and thus mimic developmental processes) an increase in COX-1 expression was observed (Smith & DeWitt, 1996). The quantitation of COX-1 in developing vasculature has indicated that the level of COX-1 isozyme increases during the 4 weeks immediately following birth (Brannon et al., 1994), and COX-1 expression was also observed to increase in seminal vesicles during puberty in response to increasing levels of androgens (Silvia et al., 1994).
Other than developmental regulation, little is known about the details of the transcriptional regulation of COX-1 gene expression (Smith et al., 1996). The glucocorticoids have little or no effects on COX-1 expression (Subbaramaiah et al., 1997). As mentioned above, the COX-1 promoter contains multiple start sites, and two SP1-type regulatory elements, and the deletion of either or both sites leads to reduction of about 50% and 75% respectively, in basal transcription of COX-1 (Smith et al., 2000).

The regulation of COX-2 expression was investigated more extensively than COX-1, in part because the high level of inducibility of COX-2 by mitogenic and inflammatory stimulation can result in easily measurable changes in mRNA and proteins (Crofford et al., 1994). The observation that rapid induction of COX-2 mRNA, which can be super-induced by cycloheximide, parallels the expression of c-fos gene, has led to the classification of COX-2 as an immediate early gene (Smith & Garavito, 1996; Smith & DeWitt, 1996). In the absence of inflammatory or other stimuli, so far COX-2 expression was only detected in a few rested tissues such as the brain (O' Neill et al., 1993; Yamagata et al., 1993; Breder et al., 1995), kidney (Harris RC et al., 1994), testes (Smith & Garavito, 1996), the pancreas (Robertson, 1998; Smith et al., 2000), stomach (Kargman et al., 1997), and the tracheal epithelium (Walenga et al., 1996). The significance of COX-2 constitutive expression is not yet fully clarified (Smith & DeWitt, 1996; Vane et al., 1998). Constitutive overexpression of COX-2 also occurs in transformed or cancerous cells, which has been observed in experimentally induced mouse epidermal papillomas and carcinomas, rat mammary carcinomas, human colon carcinomas, and human breast carcinomas (Muller et al., 1995; Kargman et al., 1995; Parrett et al., 1997; Hwang et al., 1998). However, aside from some
specialized tissues, COX-2 is normally undetectable in many resting tissues, and generally viewed as an inducible enzyme expressed transiently (induced rapidly (2-6 hours), and in most cases the protein level then decrease rapidly within 24 hours) in a variety of cells and tissues including epithelial cells, fibroblasts, monocytes, macrophages and macrophage-like cell lines, ovarian follicles, mast cells, endothelial cells, vascular and nonvascular smooth muscle cells, mesothelial cells, osteoblasts, mesangial cells, neurons, and astrocytes (Smith & DeWitt, 1996; Smith et al., 2000). COX-2 was reported to be induced in vitro, in vivo and ex-vivo, in response to pathophysiological challenges, such as growth factors, oncogenes, tumor promoters, hormones, bacterial endotoxin (LPS), serum, and cytokines. It has been shown that the factors which regulate inducible or constitutive expression of COX-2 in specialized cell types or tissues are specific for the physiological processes and tissues involved, and COX-2 expression appears to be differentially sensitive to stimuli that regulate the unique physiological activities of each tissue. For example, COX-2 expression in ovarian granulosa cells can be induced by FSH and LH, while in the kidney, overexpression of COX-2 in the macula densa depends on lumenal salt concentration (Smith et al., 2000).

The reported positive regulatory agents include PDGF, EGF, TGFα, FGF, bFGF, TNF, IL-1, IL-1β, INFγ, IgE, H₂O₂, phorbol esters (TPA, PMA), increased level of cAMP, ceramide, peroxisome proliferators, norepinephrine, serotonin, histamine, bradykinin, arginine vasopressin (AVP), angiotensin II, endothelin, serum, forskolin, Ca²⁺ ionophores, high salt, serum, parathyroid hormone, 25-hydroxycholesterol, physical stimuli such as shear force in the vasculature (which may be an important factor in eliciting the production of vascular PGI₂), UV-B radiation, LPS, thrombin, cellular transformation, and changes of
the medium such as what occurred in myocardial ischemia which leads to nonspecific release of fatty acids. The reported negative regulatory agents include glucocorticoids, retinoids, caffeic acid phenyl esters, p53, reservatol, curcumin, NSAIDs, and anti-inflammatory cytokines such as IL-10, IL-4 (Hazen et al., 1990; Evett et al., 1993; DuBois & Awad, 1994; Crofford et al., 1994; Riese et al., 1994; Xie et al., 1995; Subbaramaiah et al., 1996; 1997; 1999; Smith & Garavito, 1996; Smith & DeWitt, 1996; Sheng et al., 1998; Smith et al., 2000). Characteristically, the anti-inflammatory glucocorticoids such as dexamethasone, inhibit COX-2 expression, an inhibition as noted above that is not shared by COX-1, and such observations are considered to provide additional evidence for the critical involvement of COX-2 in inflammation.

The transcriptional regulation of COX-2 gene appears to be the major mechanism for regulation of its expression, and multiple signaling pathways have been linked to induction of COX-2 gene transcription including protein kinase A pathway, the protein kinase C pathway (phorbol esters), tyrosine kinases, viral transformation (src, ras), as well as some MAPK signaling pathways (Smith & DeWitt, 1996; Kester et al., 1994; Glaser et al., 1995; DeWitt et al., 1993; Smith et al., 2000). A post-transcriptional regulation was also reported to occur (Ristimaki et al., 1994; Newton et al., 1998). Because COX-2 mRNA was observed to be unstable compared to that of COX-1, resulting probably from the multiple RNA instability sequences present in COX-2, the factors that modulate the half life of COX-2 mRNA would also modulate the efficiency of its turnover during translation (Ristimaki et al., 1994). IL-1 appears to regulate COX-2 in part by this mechanism, as it was shown to increase the gene transcription and COX-2 mRNA stability (Ristimaki et al., 1994). On the
other hand, the anti-inflammatory steroids (e.g. cortisol, dexamethasone) were observed to inhibit the stimulated expression of COX-2 mRNA and protein (Masferrer & Reddy, 1994; Crofford et al., 1994; Newton et al., 1998) via transcriptional inhibition (DeWitt et al., 1993; Newton et al., 1998) and post-transcriptional reduction of mRNA stability (Evett et al., 1993; Newton et al., 1998). The exact mechanisms by which dexamethasone and anti-inflammatory cytokines such as IL-10 can inhibit COX-2 gene expression are not yet fully clarified (Smith & Garavito, 1996). However, the down regulation of COX-2 by dexamethasone is proposed to be mediated by suppression of NFκB and AP-1 mediated gene transcription (Subbaramaiah et al., 1996; Newton et al., 1998); and a strong effect of dexamethasone inhibition also appear to be at the level of translation, since dexamethasone not only partially inhibits COX-2 gene transcription and COX-2 mRNA accumulation, but also inhibits completely COX-2 protein expression (Rzymkiewicz et al., 1994; Newton et al., 1998).

On the metabolic regulatory aspect, COXs were reported to be regulated acutely by the level of arachidonate substrate (Smith et al., 2000), as COX-1 was shown to exhibit negative allosterism at low arachidonate concentration between 50nM and 1uM, which was not observed with COX-2; and this difference may explain the preferential metabolism of arachidonate by COX-2 in intact cells at relatively low substrate concentration (Swinney et al., 1998). The observed negative allosteric regulation of COX-1 at low concentration of arachidonate, is such that when equivalent amounts of both isozymes are functioning in this substrate range (50nM-1uM), the amount of products via COX-1 is 25% less than that formed by COX-2 (Swinney et al., 1998). This observation is of importance, since such concentration of the substrate is in the range likely to be available under many conditions.
In addition, it has been reported that the intact cells expressing both isozymes have shown preference utilization of exogenous arachidonate via COX-2 (Smith et al., 2000). The exogenous arachidonate may reach cells through normal cell-cell communications. For example, the adipocytes can supply arachidonate to neighboring endothelial cells which form PGI₂ (Parker et al., 1989). Also exogenous arachidonate may reach cells via cholesterol esters, or phospholipids of low density lipoproteins (LDL) (Habenicht et al., 1990).

**Biologic roles of COX isoforms:** A question often raised in regard to the biology of COX isozymes is why are there two isoforms?. The rationale for the existence of two isoforms is not completely known. A hypothesis of compartmentation has been proposed, based on differential gene expression, kinetic differences, the temporal patterns of the isozymes’ PGs production, subcellular localization, and coupling to different phospholipases, substrate, and different prostaglandin synthases (DuBois et al., 1998; Kulmacs et al., 1998; Smith et al., 2000). It is hypothesized that COX-1 and -2 may represent partially independent prostanoid synthetic systems. These two separate pathways may allow identical prostanoid products to play at least two different roles in signal transduction within a single cell (Smith & DeWitt, 1996; DuBois-e/ at al., 1998; Smith et al., 2000). Although both COX-1 and -2 are sometimes co-expressed in the same cell, they may act as parts of separate prostanoid signaling systems that, at least partially, function independently to channel prostanoids to the extra cellular medium (COX-1) and nuclear targets (COX-2) (Smith & Garavito, 1996; Smith & DeWitt, 1996; Kulmacs et al., 1998; Smith et al., 2000).

The first factor that may permit COXs to signal independently is the differential
gene expression, that is, constitutive vs. inducible, which suggest a functional response to specific stimuli, in cells of particular phenotypes, for the wide range more acute and transient modulation of COX-2 gene expression (Herschman, 1996). Although there are no gross kinetic differences between the COXs, the negative allosteric regulation of COX-1 at low substrate concentration permits COX-2 to oxygenate low concentration of arachidonate (<1 uM) up to four times more efficiently (Swinney et al., 1997), thus by limiting intracellular substrate concentration, arachidonate may be effectively directed to the COX-2 pathway. Also, arachidonate added at concentrations as low as 1 μM stimulate prostanoid synthesis by intact cells expressing only COX-2, whereas concentrations in excess of 10 μM must be added to stimulate prostanoid synthesis in cells expressing only COX-1, thus high concentration of arachidonate seems to favor PGs synthesis via COX-1 (Murakami et al., 1999); and this preferential channeling through COX-1 was also observed in cells that expressed both COXs (Smith et al., 2000). However, this preferential synthesis via COX-1 probably depends also on the relative levels of expression of COXs, since in many resting cells COX-2 levels typically do not reach more than 20-30% that of the constitutive COX-1 (Smith et al., 2000). Some subtle differences between the two isozymes in peroxide requirements are also reported. The estimated concentrations of hydroperoxides required to activate the cyclooxygenase activity of COX-1 were reported to be up to 10 folds higher than those required to activate COX-2 (it is estimated that COX-2 may bind hydroperoxides with 5-10 fold greater affinity than COX-1) (Kulmacz et al., 1995; 1998; Smith et al., 2000), and this difference in hydroperoxide dependency permits COX-2 to function independently of COX-1 in cells expressing both isoforms (Chen et al., 1999). In addition, the negative
allosterism of COX-1 (with low concentration of arachidonate) is not observed in the presence of excess hydroperoxide, which activate the peroxidase activity, suggesting that hydroperoxide concentration may also modulate the relative activities of COX-1 and COX-2 in vivo (Smith et al., 2000).

Depending on the model used, it appears that there is a temporal patterns of PGs production from COXs in response to stimulation (Herschman et al., 1997). Several studies have indicated a biphasic prostanoid production, with initial COX-1 dependent synthesis, followed by extended synthesis that is COX-2 dependent (Herschman et al., 1997; Smith et al., 2000). In addition, the generalized constitutive expression of COX-1 suggests that this enzyme is a part of a continuous/chronic signaling system. In contrast, the high inducibility of COX-2 suggests that this enzyme is likely to be employed in discrete/acute responses to various events. For instance, COX-2 induction occurs during (and assumed to play a key role in) hyperpolarization in nerve cells, ovulation, hypotonicity, infection and inflammation, mechanical or sheer stress, and mitogenesis (Smith & DeWitt, 1996; Smith et al., 2000).

Gross differences in the isoforms subcellular localization have not yet been established. Both COX-1 and -2 are integral membrane glycoproteins, and both appear to be present on the lumenal surface of the endoplasmic reticulum (ERet) membrane and the outer and inner membranes of the nuclear envelop (NE), but not the plasma membrane (Otto et al., 1994; Morita et al., 1995; Smith & Garavito, 1996; Kulmacs et al., 1998; Spencer et al., 1998). Although one study findings have suggested no significant difference between the nuclear envelop inner membrane/outer membrane distribution of COX-1 and -2 in the cell types analyzed (Spencer et al., 1998), several reports have concluded that COX-1 is equally
distributed between the ERet and the NE, whereas COX-2 is more preferentially associated with the nuclear envelop (Morita et al., 1995; Song et al., 1996; Taketo, 1998; Smith et al., 2000), which raises the possibility that COX-2 may have greater access to the nucleoplasm to affect nuclear events, possibly via nuclear receptors (Smith et al., 2000).

No general role has been established regarding coupling to different phospholipases that may indicate which specific phospholipase system is coupled to COX-1 or -2 (Smith et al., 2000). Although some reports have suggested selective coupling of specific phospholipase to COX-1 vs. COX-2 (Herschman, 1996, DuBois et al., 1998), the three phospholipases A2 primarily involved in agonist-induced arachidonate release have shown no general pattern of coupling, and the reported coupling may depend on the coincidental expression of PLA2s and COXs (Smith et al., 2000). However, circumstantial coupling of COXs to PLA2 system in general has been observed, which seems to depend on the amplitude of PLA2 activation by different stimuli. For example, acute (bradykinin-dependent) activation of PLA2 was shown to lead to PGE2 production via COX-1 and COX-2, whereas delayed-phase type of activation (IL-1β-dependent) produces PGE2 via COX-2 only (Smith et al., 2000). For COXs coupling to different substrate pools, experimental data from ligand-stimulated fibroblasts, macrophages, and mast cells have suggested that COX-1 and -2 may utilize different intracellular pools of arachidonic acid (Herschman et al., 1995; Herschman, 1996; Chulada et al., 1996; DuBois et al., 1998), which is likely to have functional consequences, however, the mechanisms underlying these observations are not yet clarified. A related issue is the subtle differences in COXs fatty acid substrate specificities (Laneuville et al., 1995; Smith & DeWitt, 1996; Smith et al., 2000), where the data have
indicated that COX-2, which has a larger and more accommodating cyclooxygenase active site, is more efficient in general in oxygenating the 18-carbon fatty acids than COX-1. For example, the ratio of the $K_{cat} / K_m$ value for COX-2 with $\alpha$-linolenic acid is about 40 times that of COX-1 (Smith & DeWitt, 1996).

The coupling of COXs to different PGs synthases is one unclarified aspect of prostanoid biosynthesis pathway. Considering the varied profile of eicosanoids and their sometimes contradicting functions, this step is very important in determining the outcome of agonist-induced PGs synthesis and release. COX-2 appears to be coupled preferentially to PGE$_2$ synthase, PGI$_2$, or both, as peritoneal macrophages were observed to switch from forming a mixture of PGI$_2$, TXA$_2$, PGD$_2$, and HHT to producing primarily PGE$_2$ and PGI$_2$ when COX-2 is induced by LPS (Smith et al., 2000). No precise mechanisms have been proposed yet for the apparent coupling, however, the terminal synthases may coordinate (by yet unproven mechanisms, such as protein-protein interactions, differences in kinetic properties, or the localization of the terminal synthases themselves) their expression with that of COX-2 (providing that arachidonate concentration is lower for COX-1 activation); or alternatively the selective expression of COX isozyme with a specific synthase (e.g. COX-1 and TXA$_2$ synthase in platelet) may ensure specific coupling (Smith et al., 2000).

Thus, compartmentation hypothesis suggests that COX-1 is primarily a part of an ERet prostanoid signaling system that forms prostanoids which act extra-cellularly as local hormones (intercellular mediators), functioning primarily through cell surface G-protein linked receptors, to mediate the so-called “housekeeping” responses to circulating hormones such as hemostasis, regulation of renal water and Na$^+$ metabolism, gastric cytoprotection.
and gastrointestinal secretions, and platelets functions (Hamberg et al., 1975; Smith & DeWitt, 1996; Vane et al., 1998; DuBois et al., 1998; Smith et al., 2000). Since cell membranes such as ERet are freely permeable to PGH₂, while impermeant to prostanoids other than PGH₂ (Smith et al., 1991), a prostaglandin transport systems (carriers) are more likely involved in prostanoid export process (Kanai et al., 1995). On the other hand, COX-2 expression has probably two roles, the first is a supplemental one that involves the subpopulation of COX-2 co-localizing with COX-1 on the ERet and function in the same way, that is, to generate products that end up outside the cell and function via G protein-linked receptors, thus augmenting COX-1 functions. The prostanoids formed through this action of COX-2 are proposed to play critical roles in various physiological and pathological responses. For instance, COX-2 is considered a critical player in the propagation of inflammatory responses (Smith & Garavito, 1996; Vane et al., 1998; DuBois et al., 1998; Herschman et al., 1996). The second and proposed to be a more important role involves the subset of COX-2 molecules that appear to be more concentrated on the NE membranes, which may function as part of a nuclear prostanoid signaling system, which form products that may act to regulate target genes and gene products within the nuclear envelop or the nucleus in association with cell replication and differentiation (Smith & DeWitt, 1996; Subbaramaiah et al., 1997; Smith et al., 2000). COX-2 expression is typically maximal during early stages of cell replication or differentiation, and COX-2 expression was demonstrated to occur in response to stimuli that cause cell differentiation or replication (Smith & DeWitt, 1996), events that required participation of the cell nucleus (Smith et al., 2000). In addition, studies of COXs knock out mice have suggested that each COX isozyme
may subserve unique physiological functions (Smith et al., 2000). Some investigators consider the first augmenting role as a minor one since arachidonate can diffuse freely within cells, and the gross kinetic properties ($K_m$ and $V_{max}$) of the two isozymes in regard to arachidonate are similar. In addition, it has been reported that induction of COX-2 in cells that express COX-1 constitutively appears to produce a 2-fold increase in the net prostanoid biosynthetic capacity of the cell (Smith & Garavito, 1996; Smith et al., 2000).

**COXs Gene Disruption:** Additional insights into the biologic roles of COX isozymes came from gene disruption studies. One idea behind gene disruption studies was that COXs gene knockout will provide the molecular biology equivalent to analyses based on inhibition of COX isozymes by agents such as NSAIDs. Transgenic mice in which either COX-1 and -2 genes have been inactivated were observed to display uniquely different phenotypes (Langenbach et al., 1995; Dinchuk et al., 1995; Morham et al., 1995). The COX-1 null mice lacked the gene's mRNA, protein, and enzyme activity. They survived well (suggesting that COX-1 expression does not appear to be critically required for development), showed reduced platelet aggregation, and exhibit no gastric or intestinal pathology nor any renal dysfunction, and showed less indomethacin-induced gastric ulceration than the wild-type mice (Langenbach et al., 1995; Williams & DuBois, 1996; Vane et al., 1998). They have also decreased inflammatory responses to arachidonic acid, but not phorbol ester stimulation (Langenbach et al., 1995; Vane et al., 1998). COX-1 null mice produced few life offspring, suggesting that COX-1 is apparently essential for fetuses’ survival (Vane et al., 1998), however, this observation was also attributed to parturition difficulties in the COX-1 null female mice (Williams & DuBois, 1996).
Unlike COX-1, COX-2 expression appears to be required for proper development (Morham et al., 1995; Vane et al., 1998). In the COX-2 null mice, several developmental defects were observed, such as the absence of the corpora lutea in the developing ovary (indicating no ovulation and infertility); renal nephropathy (which was severe enough to produce shorter life span); cardiac fibrosis; and increased susceptibility to peritonitis (Morham et al., 1995; Dinchuk et al., 1995), suggesting teratological potential of COX-2 inhibition (Vane et al., 1998). The COX-2 null mice were shown to have normal platelet function, and to be as susceptible to inflammation as the wild-type animals, which was surprising, given the role assumed to be contributed by COX-2 in inflammation (Wu K et al., 1996). The COX-2 null mice have shown unchanged (normal) inflammatory responses to acute experimental inflammation induced by arachidonate or phorbol esters, suggesting that the inhibition of COX-2 derived PGs synthesis may not be the sole factor in the anti-inflammatory mechanisms of NSAIDs; and that the physiological effects of eliminating a COX isozyme are not equivalent to those caused by its interaction with NSAIDs (Morham et al., 1995). For the first observation, an explanation provided was that the inflammatory reaction is a complex process involving many mediators of inflammation, and the measurements of the inflammatory responses in those experiments have occurred within the first four hours of stimulus application and could have been due COX-1 derived PGs (Vane et al., 1998; Taketo, 1998). An alternative hypothesis proposed for the second observation has involved the interaction of NSAIDs with the enzyme in a way that generate aberrant enzymatic products, either due to alteration in the arachidonate metabolic pathway (such as the production of 15R-HETE from the interaction of aspirin and COX-2, where 15R-HETE
can be further metabolized into bioactive lipoxins by the lipooxygenase pathway), or due to blockage of cyclooxygenase active site in a manner that cause aberrant usage of the hydroperoxidase active site (Morham et al., 1995; Taketo, 1998). It is worthy to note that gene disruption experiments are complicated by the fact that different phenotypes have been observed for disrupting identical genes in different genetic backgrounds such as different strains of mice (Williams & DuBois, 1996), and caution should be generally applied in the interpretation of results from the knockout models (Vane et al., 1998).

1.4.2. The Eicosanoids.

The eicosanoids (eikosi = twenty), also known as the autocoids (exert their effects primarily in the tissue in which they are produced) are the products of metabolism of C₂₀ polyunsaturated fatty acids containing 3, 4, or 5 carbon-carbon double bonds, such as eicosatrienoic acid (C₂₀:₃), also known as dihomo-γ-linoleic acid; eicosatetraenoic acid (C₂₀:₄), also known as arachidonic acid; and eicosapentaenoic acid (C₂₀:₅), also known as EPA (Glew, 1992). The C₂₀:₃ fatty acid is the precursor to the 1-series of prostanoids such as PGE₁, the C₂₀:₄ is the precursor of the 2-series of prostanoids such as PGE₂, and the C₂₀:₅ is the precursor of the 3-series of prostanoids such as PGE₃ (Glew, 1992). Thus, the type of the substrate upon which COX isozymes may act would determine which series of prostanoids is produced, and that may explain in part the sometime contradicting functions of this metabolic pathway products. Since most of arachidonic acid (C₂₀:₄) metabolites contain 20 carbon atoms, they are generally referred to as eicosanoids. The compounds of the 2-series (derived from arachidonic acid) such as PGE₂, PGF₂, and TXA₂ are the principal prostanoids in humans and are of the greatest significance biologically (Lupulescu, 1996).
The eicosanoids can be generally categorized into three categories, depending on the pathway of their formation from arachidonic acid. These pathways are: {1} The bis-dioxygenated (2O₂) fatty acids' derivatives, the prostanoids which comprise prostaglandins (PGs), and thromboxanes (TXs). This pathway is catalyzed by the COX isozymes. The prostanoids are all related or analogues to prostanolic acid, which does not occur naturally, but considered the parent compound of prostaglandins for the purpose of nomenclature and carbon numbering (Glew, 1992; Davidson, 1994). {2} The mono-dioxygenated (O₁) fatty acids' derivatives which comprise: [a] the hydro-peroxy-eicosa-tetra-enoic acids (HPETEs), which are hydroxy fatty acid derivatives that do not contain a ring structure, and are converted to either the active compounds, leukotrienes (LTs), or the hydroxy fatty acids (HETEs, and diHETEs), and [b] the Lipoxins (LXs), and hepoxilins. This pathway is catalyzed by the enzymes 5,12, and 15 lipoxygenases (LOXs). It is not yet completely clear whether HPETEs are biologically active compounds (Davidson, 1994). {3} The mono-oxygenated (O) fatty acids' derivatives, the arachidonic acid epoxides and diols: [a] the epoxy acids (EETs), [b] the dihydroxy acids (DHETs), and [c] ω-Hydroxy fatty acids. This pathway is catalyzed by the P-450 epoxygenases (Smith et al., 1991; Capdevila et al., 1992; Subbaramaiah et al., 1997; Smith et al., 2000). In addition, there are the isoprostanes, isoleukotrienes, and other peroxidized fatty acid products that are formed non enzymatically (Smith et al., 2000).

The most important dietary precursor of eicosanoids is the essential fatty acid linoleic acid (C18:2). A minor part of the total intake of linoleic acid is converted, primarily in the liver, by elongation and desaturation to arachidonic acid (C20:4), and to some extent
to dihomo-γ-linoleic (C20:3) and EPA (C20:5) (Glew, 1992; Laneuville et al., 1995). The amount of adults' linoleic acid intake is estimated to be 10 grams/day, and the total excretion of eicosanoids and their metabolites is reported to be about 1 mg/day, suggesting that the eicosanoid metabolism represents a quantitatively minor part in the overall fatty acid's metabolism, however, eicosanoids metabolism depends to great extent on a constant and regular intake of linoleic acid, since a diet deficient in linoleic acid has been observed to cause decreased production of eicosanoids (Glew, 1992).

The observed best two fatty acid substrates for the cyclooxygenase activity of both COX isozymes are the twenty carbon ω6 (n-6) polyunsaturated fatty acid derivatives of linoleic acid (18:2) such as arachidonic acid (20:4) and dihomo-γ-linoleic acid (20:3) (Smith & DeWitt, 1996). COXs can also utilize the ω3 (n-3) polyunsaturated fatty acid derivative EPA (20:5) (converted to PGH3), the so called fish oil fatty acid, but the rate of catalysis is relatively low (Smith & DeWitt, 1996). This may account in part for the putative beneficial effects of including fish oil in the diet, where the long chain ω3 (n-3) fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were observed to exert a protective effect against breast cancer (Rose, 1997). Also several epidemiological studies have associated high olive oil (rich in the ω9 fatty acid, oleic acid) consumption with a relatively low breast cancer incidence (Rose, 1997).

COXs are also capable of catalyzing the oxygenation of linoleic acid, α-linolenic and γ-linolenic acids, where these 18-carbon fatty acids are converted to monohydroxy acids (Laneuville et al., 1995). Although the ω3 (n-3) and ω9 (n-9) polyunsaturated fatty acids containing 18-22 carbons are considered poor substrates for COXs, they were reported to be
efficient competitive inhibitors of arachidonate oxygenation. For example, Docosahexaenoic acid (22:6, ω3) is a competitive inhibitor of both COXs without being a substrate for either isozyme (Meade et al., 1993). In rodent models of mammary carcinogenesis, the level and type of dietary fat seem to influence the development and growth of spontaneous, transplantable, and chemically induced tumors (Ramchurren et al., 1995; Rose, 1997).

**Prostanoids Metabolism:** The regulation of prostanoids’ biosynthesis and release is a complex process, and the precise mechanisms that govern their biosynthesis and release are not fully understood. The prostanoid metabolic pathway can be characterized into three phases: {1} the mobilization (release) of the arachidonate substrate; {2} the conversion (cyclization/oxygenation) of arachidonate to the PGG, then (reduction) to PGH; and {3} the conversion (rearrangement/reduction) of PGH to the biologically active PGs and TXs (Smith et al., 1991; Glew, 1992). An additional phase, is {4} the deactivation phase. The eicosanoids cascade is illustrated in figure 1.2.
Figure 1.2 Arachidonate metabolism via COX and LOX pathways.
PG, prostaglandin; HPETE, hydroperoxy eicosatetraenoic acid; LT, Leukotriene
The Substrates Mobilization (Release): In mammalian cells the two major pathways of C_30 fatty acids metabolism that produce prostanoids and leukotrienes are generally known as the cyclooxygenase (COX) and the lipooxygenase (LOX) pathways. The main substrate for both pathways is the unesterified twenty-carbon monocarboxylic acid, arachidonic acid. It is not yet known with confidence whether the arachidonate used to synthesize these two groups of compounds come from different precursor pools, or whether certain phospholipases are involved in either pathway. It is known, however, that inhibition of COX, by aspirin for example, would lead to increase in production of LOX pathway products, indicating that arachidonate mobilized for prostanoid production can be channeled to the LOX pathway under certain pharmacological conditions (Glew, 1992). In addition to being the primary substrate for COXs, arachidonic acid is also a second messenger (Blobe et al., 1995). In cell membranes, the polyunsaturated fatty acids such as arachidonic acid are usually (almost exclusively) esterified to the position 2 (sn-2) of the glycerol moiety of phospholipids (Subbaramaiah et al., 1997).

In response to extra-cellular stimulation of a target cell with relevant stimuli (such as a cytokine, growth factor, or circulating hormone), arachidonate can be mobilized from membrane phospholipids such as phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS), by the action of hydrolases such as phospholipase A_2 (primarily), and the PI-specific phospholipase C (Smith & DeWitt, 1996). Some experimental studies have also implicated phospholipase D (Pouliot et al., 1998), which was initially linked through diglyceride (DAG) formation to PKC signaling pathway, and recently linked with phosphatidic acid as the relevant signaling molecule in
some signaling pathways that lead to stimulation of COX-2 (Daniel et al., 1999). This step of esterolytic cleavage of lipids by the phospholipases is the rate-limiting step in eicosanoid biosynthesis (Smith et al., 1991; Glew, 1992; Smith et al., 2000). The two classes of phospholipids, PC and PI, are considered to be the main contributors of arachidonate. In addition, the triglycerides or cholesterol esters containing arachidonic acid may also be used as a source of arachidonate substrate (Smith et al., 1991; Glew, 1992; Smith & DeWitt, 1996; Daniel et al., 1999).

The stimulatory effects of some agent(s) on prostanoids production may be partially mediated through activation of one or more specific phospholipases (Murayama et al., 1990; Smith et al., 1991), however, the precise mechanisms by which extracellular stimuli activate phospholipases (which consist of groups of closely related isoforms), remain unclear (Smith & DeWitt, 1996; Blobe et al., 1995). Most of the evidence suggests that arachidonate release occur through the action of phospholipases A₂ which are considered quantitatively to be the most important pathway (Bettazzoli et al., 1990; Smith & Garavito, 1996). Three phospholipase A₂ (PLA₂) isoforms are primarily involved in agonist-stimulated arachidonate release: type IV cytosolic (c)PLA₂ (which can be activated by several signaling pathways which result in activation of the MAP kinases or intracellular Ca²⁺ increase), and type IIA and V secreted (s)PLA₂ enzymes (Anderson et al., 1997; Smith et al., 2000). The activation of phospholipases A₂ can be characterized into early phase (ionophores-stimulated) and late phase (LPS-stimulated), and it seems that there is a hierarchy to the activation of PLA₂s involving a particular dependence on cPLA₂, since both phases of activation were abrogated in peritoneal macrophages from cPLA₂-knockout mice (Smith et al., 2000). The regulation
of phospholipases A₂ is quite complex, and may involve control at both the transcription and
translation levels (Davidson et al., 1990; Bailey et al., 1991; Glaser et al., 1995). They were
shown to be inhibited by several agents such as the glucocorticoids, and NSAIDS (Bailey et
al., 1991; Vervoordeldonk et al., 1996).

An extracellular stimuli receptors can also be linked to \( G_{\text{PLC}} \) proteins, which when
activated can activate phospholipase C that in turn hydrolyzes (PI) into inositol triphosphate
\((\text{IP}_3)\) and diacylglycerol (DAG), both of which are considered second messengers (Wellman
et al., 1994). In many cells, \( \text{IP}_3 \) increases the intracellular free Ca\(^{2+}\) concentration, needed
for DAG activation of the Ca\(^{2+}\) - dependent isoforms of protein kinase C (PKC) (Wellman
et al., 1994). The tumor promotors, phorbol esters, are thought to mimic the effects of DAG,
and since they are not rapidly hydrolyzed like DAG they result in prolonged activation of
PKC, which is implicated in modulating cellular proliferation and differentiation through its
various isozymes (Wellman et al., 1994; Boorne et al., 1998).

The cell that have been studied most in regard to arachidonate release is the human
platelet. In platelets the thrombin-induced arachidonate release is selective, i.e., there is no
appreciable release of linoleate or oleate (Smith et al., 1991). In platelets, arachidonic acid
is the major intracellular free fatty acid with concentration in the low uM range. Partitioning
studies have revealed that almost 70% of arachidonate was found in the cytosol, with only
30% partitioning into the membranes (Blobe et al., 1995). In contrast, for oleic acid (OA),
which is a major extracellular free fatty acid found circulating in the blood at concentrations
in the 100-150 uM range, partitioning studies have revealed that 70% of OA rapidly
partitioning to the membrane and 30% remained in the cytosol (Blobe et al., 1995). These

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differences were attributed to the presence of fatty acid binding proteins (FABP) which are able to increase the solubility of free fatty acids often by several orders of magnitude, and may selectively or preferentially allow for the high concentration of cytosolic arachidonic acid to exist in vivo (Blobe et al., 1995).

(2)- COX catalysis: The initial step in biosynthesis of prostanoids is accomplished by the central COX isozymes, whose catalyzed reactions are found in cellular membranes of the ER, and NE (Smith et al., 1996; Smith & DeWitt, 1996; Smith et al., 2000). The substrates for the cyclooxygenase reaction are two molecules of oxygen and one molecule of fatty acid, thus, arachidonate can be viewed as the storage form of prostanoids (Smith & DeWitt, 1996). In the cyclooxygenase reaction arachidonate is converted (oxygenated and cyclized) to PHG, which then released from the cyclooxygenase active site to the spatially distinct peroxidase active site where it undergoes reduction to PGH in the peroxidase reaction (Horton et al., 1993; Davidson et al., 1994; Tsai et al., 1995; Smith & Garavito, 1996; Smith et al., 1991; 2000). COX-1 which primarily synthesize PGG, can also produce significant amounts of 11R-HETE, 15S-HETE, and 15R-HETE; whereas COX-2 can produce the same compounds except 15R-HETE, suggesting that arachidonate may exist as slightly different but catalytically competent conformers in the cyclooxygenase active site (Smith et al., 2000).

(3)- PGs - Specific Synthases Catalysis: Once formed, PGH is converted by different relevant specific enzymes (PGs Synthases) such as PGE Synthase (PGES), PGF Synthase (PGFS), PGI Synthase (PGIS), to produce several prostaglandins and thromboxane (Vane et al., 1998; DuBois et al., 1998; Smith et al., 2000). The details of these additional steps leading to the individual prostaglandins remain to be fully elucidated. The formation of
primary prostanoids by these specific synthases whose expression varies depending on tissue and cell types, results in a degree of tissue specificity as to the type and quantity of the prostanoid produced (Glew, 1992). For example, in the kidney and spleen, the PGE$_2$ and PGF$_{2\alpha}$ were reported to be the major prostanoids produced, whereas in the blood vessels PGI$_2$ is the mostly produced prostaglandin. In the heart PGE$_2$, PGF$_2\alpha$, and PGI$_2$ were observed to form in approximately equal amounts, whereas thromboxane A$_2$ (TXA$_2$) is the main prostanoid formed in platelet (Glew, 1992).

PGE$_1$ and PGE$_2$ can be formed by PGES enzyme-mediated non oxidative rearrangement of PGH$_1$ and PGH$_2$, respectively. PGE derivatives can also be formed non enzymatically at relatively rapid rate from PGH precursors. There appears to be a number of proteins (isoenzymes) which exhibit PGES activity in different tissues, while there are two reported forms of PGF which exhibit biological activity, PGF$_{2\alpha}$ and 9$_{\alpha}$,11$\beta$-PGF$_2$, and the PGF derivatives appear to be the only immediate metabolites of PGH that are formed by reduction, while PGE, PGD, PGI, and TXA are all reported to be synthesized by non oxidative rearrangements (Smith et al., 1991). PGFS catalyzes, probably at different sites, the formation of PGF$_{2\alpha}$ and 9$_{\alpha}$,11$\beta$-PGF$_2$, using preferably NADPH as a reducing agent, and PGF$_2$ derivatives can also be formed from reduction of PGE$_2$ by different reductases (Smith et al., 1991; Forman et al., 1995).

PGD$_2$, a major COXs product widely distributed in body tissues, is formed by a PGDS enzyme-mediated rearrangement of PGH$_2$, and there appear to be at least three different proteins (isoenzymes) which exhibit PGD synthase activity (Smith et al., 1991). PGD$_2$ was shown to undergo dehydration readily in vivo and in vitro to yield additional, biological
active PGs of the J series, the ultimate metabolites of PGD₂ (Fukushima et al., 1992; Kliwer et al., 1995). Prostaglandin A series were also reported to be dehydration (spontaneous or protein catalyzed) products of the PGD series (Fukushima et al., 1992; Marnett, 1992).

PGI (prostacyclin) is catalyzed by PGIS from PGH₂ and PGH₃ which were shown to serve as substrate for PGIS, but not PGH₁; and high PGIS concentration has been reported in vascular endothelial cells and in both vascular and nonvascular smooth muscle cells (Smith et al., 1991). Coupling of COX-2 to PGI₃ synthase in the cardiovascular system has been proposed, where secretion of prostacyclin (but not thromboxane metabolites) was reported to be reduced by 70-80% in volunteers given anti-inflammatory doses of COX-2 inhibitors such as Celebrex and Vioxx, which suggests that COX-2 may be an important source of vascular and probably renal prostacyclin (Smith et al., 2000).

TXA₂, synthesized by lungs and macrophages in addition to platelets, is a potent thrombogenic and vasoconstrictor agent, and TXA₂ formation is catalyzed by the thromboxane A synthase (TXAS) (Smith et al., 1991; Glew, 1992). It is worthy to note that formation of TXA₂ from PGH₂ is always accompanied by the formation of at least an equivalent amount of HETrE (also called HHT), and malondialdehyde (MDA); and with PGH analogs other than PGH₂, the amounts of HETrE and MDA formed are even far in excess of TXA₂ (Smith et al., 1991; Marnett, 1992). A relevant point to mention is that MDA was shown to be mutagenic in mammalian test systems (Marnett, 1992), and HETrE was shown to be an angiogenic factor (Weidner et al., 1996).

{4}- Prostaglandins Deactivation: The prostanoids were shown to have a very short half-
and soon after release they are rapidly taken up by cells and then inactivated. For instance, TXA\(_2\) which is produced in abundant in lung cells and platelet, has a very short half-life in water (\(t_{1/2}\) of TXA\(_2\) is \(\frac{1}{2}\) -1 minute) and is transformed (hydrolyzed) rapidly into the stable but biologically inactive TXB\(_2\) (Glew, 1992). In breast cancer tissue, PGE\(_2\) produced by breast fibroblasts, was shown to be rapidly and greatly reduced due to metabolic inactivation by cancerous epithelial cells which convert PGE\(_2\) to 15-keto-PGE\(_2\) by the enzyme 15-hydroxy-PG dehydrogenase (PG dehydrogenase), which is considered to be the key enzyme controlling the biological inactivation of prostaglandins (Xun et al., 1991; Schrey et al., 1995). Therefore, factors that regulates this enzyme may determine the levels of PGE\(_2\) in breast cancer tissue. This enzyme was reported to be inhibited by estrogen, in contrast to progestins which were shown to induce the enzyme; and antiprogestins were shown to be effective in suppressing the enzyme activity and elevating in situ PGs concentrations (Lupulescu, 1996; Cheng et al., 1993). This enzyme was also shown to be induced by protein kinase C-activating phorbol esters which induce de novo enzyme synthesis and activity, and was inhibited by cycloheximide treatment (Xun et al., 1991).

**PGs Receptors:** Hormones can be generally classified by three schemes: (1) by the proximity of their site of synthesis to their site of action; (2) by their chemical structure; and (3) by their degree of water solubility (Wellman et al., 1994). The prostanoids, which are fatty acids derivatives, are considered to have paracrine and autocrine effects (via a carrier mediated process) (Chan BS et al., 1998), and to elicit their effects on target cells via G protein-linked prostanoid receptors (Ushikubi et al., 1998; Sugimoto et al., 1998), or in some cases may interact with nuclear receptors (Lim et al., 1999). This difference in solubility in
the cytosol most likely reflects the effect of fatty acids binding proteins (FABP) (Blobe et al., 1995). The typical responses of a cell to a prostanoid receptor activation include changes (increase/ decrease) in the intracellular concentration of cAMP (through activation/ inactivation of adenylyl cyclase) and stimulation of Ca$^{2+}$ mobilization, or both (Sonnenburg et al., 1990; Smith et al., 1991). Some of the proposed inhibitory PGs such as PGA analogues and PGJ$_2$ were reported to inhibit DNA synthesis and cell proliferation in fibroblasts, murine neuroblastoma, and human mammary tumors independently of cAMP (Lupulescu, 1996).

Cellular responses to prostanoids, thus, appear to be mediated through two classes of receptors, the specific G protein-coupled cytoplasmic receptors (with seven transmembrane domains) class such as EP for PGE$_2$ (4 subtypes reported), which transduce signals upon binding of ligands by modulating the levels of second messengers; and the nuclear PPARs receptor class such as PPAR$_{\alpha}$, PPAR$_{\gamma}$, PPAR$\delta$, which acts directly as nuclear transcription factors upon ligand binding (Hirata et al., 1994; Johnson et al., 1996; Lupulescu, 1996; Tai et al., 1997; DuBois et al., 1998). Also, some of the known splice variants of the cytoplasmic membrane receptors may be targeted to the nuclear envelop (Nampa et al., 1993). There appears to be a distinct set of the first class of PGs receptors for each of the prostanoids, which are classified as DP, EP, FP, IP, and TP according to the type of PG, i.e. D, E, F, I (prostacyclin), and T (thromboxanes). Also, different subsets were isolated (Gusovsky et al., 1991; Lupulescu, 1996; Smith & DeWitt, 1996).

On the other hand, PPARs are members of nuclear receptor superfamily that include receptors for steroid, thyroid, and the retinoid hormones (Forman et al., 1995). The PPARs
appear to bind as heterodimers with the retinoic X receptor (RXR) to the peroxisome proliferator response element in the targeted genes and regulate their expression as a transcription factor complex (Kliewer et al., 1995; Johnson et al., 1996). The precise role of this class of receptors in carcinogenesis remains to be clarified. Some experimental data have indicated that chronic exposure to peroxisome proliferators leads to the development of hyperplasia and tumors in some animal species (Johnson et al., 1996), and PPARβ, which is reported to be regulated by the APC tumor suppressor gene, was shown to be overexpressed in colon cancer cells, and NSAIDs such as sulindac were observed to inhibit its activity through disruption of DNA binding ability of PPARβ/RXRα heterodimers (He et al., 1999; Wu et al., 2000). On the other hand, peroxisomal proliferator-activated receptor γ (PPAR-γ) has been shown to be activated by the proposed inhibitory cyclopentenone prostaglandins such as PGJ₂ (Forman et al., 1995).

**Leukotrienes Metabolism:** Another biologically important pathway of arachidonate metabolism is the lipooxygenase (LOX) pathway, which catalyzes the mono-oxygenation of polyunsaturated fatty acids such as arachidonic acid to hydroperoxides, designated HPETEs (for mono-hydroperoxy-eicosatetraenoic acids) (Glew, 1992). There is a family of lipooxygenases, the most important are the 5, 12, and 15 LOXs, and specific stimuli or signals determine which type of lipooxygenase product is produced by a given type of cells. The 5-HPETEs are the major products in tissues undergoing inflammatory responses (Glew, 1992). The HPETEs are highly unstable intermediates that are converted either to hydroxy fatty acids (alcohols) such as HETEs by the action of peroxidases; or hydrolyzed to

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leukotrienes such as the epoxide LTA₄, by the action of LTA₄ synthase. LTA₄ can be converted to the diol LTB₄ by the action of LTB₄ synthase; or can be further converted into LTC₄, LTD₄, LTE₄, through the actions of specific peptidases (Glew, 1992). The lipooxygenase pathway products are reported to be potent mediators of processes involved in inflammation and allergic (hypersensitivity) reactions, cellular secretion of hormones such as insulin, cell growth, cell movement, and calcium fluxes (Lee et al., 1992). In general, the HETEs, particularly the 5-HETE, and LTB₄ are involved in the regulation of leukocytes and other inflammatory cells functions, and act as mediators of chemotaxis, stimulation of adenylate cyclase, and the release of lysosomal hydrolytic enzymes. The leukotriene LTD₄ is referred to as the slow-reacting substance of anaphylaxis (SRS-A) which causes smooth muscle contraction and is about 1000 times more potent than histamine in constricting the pulmonary airways, and it causes changes in microvasculature permeability that leads to edema (Abbas et al., 1991; Davidson et al., 1994). The products of the lipooxygenase pathway have also been implicated in cancer development, including mammary carcinogenesis (Rose et al., 1997).

1.4.3. Roles of COXs / Prostaglandins in Physiopathology and Cancer:

In spite of their structural complexity and the diversity of their sometimes conflicting functions, the prostanoids’ potent physiological and pharmacological effects have given them an important place in biology and medicine. The prostaglandins were initially recognized through their effects on smooth muscles, specifically their ability to promote the contraction of intestinal and uterine muscles and lowering of blood pressure (Glew, 1992). With the
exception of red blood cells, the prostanoids are produced and released by nearly all mammalian cells. Unlike other hormones, prostanoids are not stored in cells, but instead are believed to be synthesized and released promptly to act as local hormones, at or near their sites of synthesis (Smith et al., 1991; Glew, 1992).

**Roles in Physiopathology:** The prostanoids, widely distributed in tissues at very low concentration, act as critical mediators in a variety of physiological processes, however, their precise roles are not fully understood, and sometimes appear conflicting depending on the type of the cell/tissue and the stimulus/ligand. The different effects of the PGs can be explained, however, by considering their varied chemistry, the diversity of PG receptors, and modulation of PG synthesis by local upstream and downstream effects (Glew, 1992; Davidson et al., 1994; DuBois et al., 1998). The prostaglandins are natural mediators of inflammation, and administration of prostaglandins such as PGE₂ for example, have been reported to induce signs of inflammation including redness, heat, edema and swelling (probably due to arteriolar vasodilatation and increased capillary permeability). The COX-2 isozyme which is particularly responsive to and most often elevated by growth factors and mediators of inflammation such as IL-1, TNFα and LPS, and suppressed by glucocorticoids and anti-inflammatory cytokines, now appears to be the relevant isozyme in inflammatory reactions (Masferrer et al., 1994; Smith CJ et al., 1998; DuBois et al., 1998; Smith et al., 2000). Some of the evidence have indicated that COX-1 also contribute to the inflammatory reactions, but the exact role of COX-1 in the inflammatory process remains to be clarified (Wallace, 1999). The inflammatory reactions are frequently treated with corticosteroids that inhibit COX-2 derived prostaglandin synthesis, however, some NSAIDs such as peroxicam
have shown potent anti-inflammatory effects but have little affinities for COX-2 (Kulmacz \textit{et al.}, 1994). One proposed explanation has postulated that the inhibition of COX-1 by such agents may have the net effect of suppressing cAMP formation, and that in some inflammatory situations cAMP promote the expression of COX-2 (Smith & DeWitt, 1996). NSAIDs that selectively inhibit COX-2 were shown to have potent anti-inflammatory activities in humans, whereas no corresponding studies with selective COX-1 inhibitors have been reported, probably due to concerns about side effects.

The prostaglandins are also mediators of \textit{pain and fever}, and COX-2 seems to play an important role in these processes (DuBois \textit{et al.}, 1998; Vane \textit{et al.}, 1998). Administering PGE\(_2\), in amount that alone do not cause pain, before the administration of the autacoids histamine or bradykinin, was shown to enhance both the intensity and the duration of pain caused by these two agents (Glew, 1992). It was also proposed that activation of PGs synthetic pathway by pyrogen may result in the release of PGE\(_2\) in the region of the hypothalamus where body temperature is regulated, and NSAIDs such as aspirin may exert its antipyretic effects by inhibiting COXs and PGs production (Vane \textit{et al.}, 1998). PGs such as PGD\(_2\), have marked effects on \textit{nerve cell functions} and sleep regulation, and COX-2 is reported to be an important modulator of neural responses (DuBois \textit{et al.}, 1998). PGD\(_2\) has also been shown to be released by mast calls, suggesting a role in \textit{immunologic processes} (Smith \textit{et al.}, 1991; Kliewer \textit{et al.}, 1995; Herschman \textit{et al.}, 1997).

The prostaglandins seem to play an important role in controlling \textit{blood vessels tone} and arterial pressure (Wu, 1996; DuBois \textit{et al.}, 1998), and it has been reported that a significant proportion (>80%) of PGI\(_2\) production in healthy individuals occurs via COX-2.
(McAdam et al., 1999). The vasodilatory prostaglandins, derived from both COXs, such as PGE, PGA, PGD and PGI₂ (prostacyclin) were shown to lower systemic arterial pressure by decreasing peripheral resistance, thereby increasing local blood flow (Kerins et al., 1991; Fukushima et al., 1992). The COX-1 appears to be important participant in producing vasodilating PGs in the kidneys under contractile conditions (DuBois et al., 1998). On the other hand, a COX-1 product, TXA₂ (produced by platelets) is a vasoconstrictive prostanoid which causes contraction of the vascular smooth muscles and glomerular mesangium (Subbaramaiah et al., 1997). Certain PGs, namely PGI₂, were observed to inhibit platelet aggregation, whereas PGE₂ and particularly TXA₂ promote this clotting process (Glew, 1992; DuBois et al., 1998) and one pathology associated with COX-1 is thrombosis (Patrano et al., 1994). Thus platelet-derived TXA₂ and endothelial-derived PGI₂ seem to act as antagonists and have a local balanced relationship which play a role in the control of local thrombosis. The synthesis of endothelial cell-derived PGI₂ appears to be predominantly dependent on COX-2 activity (Mandell, 1999).

The PGs are important in maintaining normal gastrointestinal functions, presumably through regulation of secretions as well as mucosal blood flow (Masferrer et al., 1996; Subbaramaiah et al., 1997; DuBois et al., 1998; Vane et al., 1998). Inhibition of the vasodilating PGs was proposed to alter the blood flow in the micro circulation of gastric mucosa leading to ischemia followed by mucosal damage and ulceration, and COX-1 inhibition (e.g. by NSAIDs) is proposed to be the major underlying mechanism (DuBois et al., 1998; Vane et al., 1998). On the other hand, the PGs were observed to accelerate the healing of gastric ulcers, and a synthetic PGE₂ was reported to be very effective in inhibiting
gastric acid secretion in patients with peptic ulcers, and the inhibitory effect appeared to be due to inhibition of cAMP formation in the gastric mucosal cells (Glew, 1992).

The prostanoids produced by COXs are important participants in the regulation of renal functions (DuBois et al., 1998; Vane et al., 1998; Brater, 1999). In the collecting tubules and juxtaglomerular (JG) apparatus, they appear to be involved in the regulation of water retention, sodium resorption, and potassium secretion, as well as renin secretion, thus participating in the regulation of salt and volume homeostasis, and renal blood flow (Smith & DeWitt, 1996; Subbaramaiah, 1997; Brater, 1999). It is proposed that PGs do not maintain normal renal blood flow, but PG production becomes critical in maintaining blood flow of the compromised kidney, and the predominant PG therein is PGI₂ (Vane et al., 1998; Brater, 1999). Although the details of the interactions between COX-1 and -2 mediated systems in the kidney are not yet clear, COX-2 seems to have a distinct role (DuBois et al., 1998; Vane et al., 1998).

Prostanoids such as PGE₂ and PGF₂α are important in reproduction, ovulation, luteinization, sperm migration, fertilization, implantation, fetal development, and partition as smooth muscle contractors (Wu, 1996; Subbaramaiah et al., 1997; DuBois et al., 1998; Vane et al., 1998). Induction of COX-2 expression was reported to be involved in several stages of mammalian reproduction. For example, gonadotrophins were observed to stimulate induction of COX-2 expression in ovarian follicles proceeding ovulation, and the induction of COX-2 appears to be a prerequisite for ovulation, at least in rodents (Sirois et al., 1994). COX-2 was also reported to be induced in differentiating uterine stromal cells at the sites of the blastocyst implantation, suggesting participation of COX-2 in the cell growth and
neovascularization that accompany this process (Jacobs et al., 1994). Furthermore, the signaling from COX-2-derived PGI₂ (prostacyclin) seems to be important for embryonic implantation, since no ova was observed to implant in COX-2 knockout mice, and the addition of a synthetic prostacyclin analogue was shown to allow implantation to proceed in that COX-2 deficient mice (Smith et al., 2000). There were no PGI₂ cell surface receptors detected in the implantation site, suggesting that PGI₂ signaling may be mediated through the PPAR δ nuclear receptor (Lim et al., 2000). Increased PGs synthesis appears to be also required for parturition, as levels of COX-2 from the amnion were observed to be twice as high in spontaneous labor compared to selective cesarian section, suggesting that prostanoid synthesis at normal parturition may be entirely through COX-2 (Hirst et al., 1995). One of the functions of PGE series in the fetus is to maintain the patency of the ductus arteriosus prior to birth, and its closure cold be hastened by the administration of COX inhibitors if the ductus remains open after birth. However in case of infants born with congenital abnormalities that can be corrected surgically, infusion of PGs will maintain the blood flow through the ductus over the interim period (Glew, 1992).

The prostanoids may be further subdivided into two general groups, conventional and cyclopentenone-type prostanoids, according to their observed actions (Odani et al., 1996). The cyclopentenone PGs, such as the PGA and PGJ₂ series are proposed to exert a variety of biological actions including inhibition of cell growth, and promotion of cell differentiation (Odani et al., 1996). The PGA₁ and PGA₂ were reported to have antiproliferative effects on many cell lines including breast cancer cell lines (Fukushima et al., 1992), and it has been suggested that the effects of PGA₁ and other antitumor PGs may
partly be due to their immuno stimulatory activity at lower doses (Fukushima et al., 1992). Also the observed G₁ growth arrest by PGA₂ was shown to be mediated through the induction of the cyclin-dependent kinase (cdk) inhibitor p21^{WAF} protein independent of the p53 status (Gorospe et al., 1996). PGD₂ was reported to cause inhibition of c-myc oncogene expression, and DNA synthesis, however, the antiproliferative effects of PGD₂ may be interpreted as mediated by PGJ₁ (Fukushima et al., 1992). Several studies have reported that PGD₂ and PGJ₁ and its metabolites to be potent activators of PPARα and PPAR γ; and that PGJ₁ and its metabolites to be endogenous natural ligands and agonists for PPAR γ, which is often expressed at high levels in adipose cells, thus, they may act as adipogenic agents (Kliewer et al., 1995; Forman et al., 1995; Johnson et al., 1996). PGJ₁ metabolites were also shown to induce cell cycle arrest and apoptosis through PPAR γ-independent pathway (Forman et al., 1995). The cyclopentenone PGs of the A and J series have also been shown to exert antiviral activities in vitro as well as in vivo with a variety of both RNA and DNA viruses (Fukushima et al., 1992). These cyclopentenone PGs were observed to be produced in response to various stress stimuli, and are actively (through specific fatty acids binding carrier proteins) incorporated into cells and transferred to the nucleus (Fukushima et al., 1992), where they were shown to activate the gene expression of various stress-related proteins such as ribosome-inactivating protein, heme oxygenase, and those of the cytosolic heat shock proteins (HSPs) such as the BiP gene whose functions are considered to be important for cellular protein homeostasis because of its involvement in translocation, folding and assembly of nascent proteins (Odani et al., 1996). The heat shock proteins (HSPs) were also shown to cause cell arrest at G₁ phase (Fukushima et al., 1992).
**Roles in Cancer:** The roles of COX-1 in carcinogenesis, including mammary cancer, are not yet clarified. COX-1 was reported to be expressed in both normal and neoplastic regions in many tissues, including mammary tissues (Hwang et al., 1998), but seems to be particularly expressed in the tumor stroma that include fibroblasts, smooth muscle cells, and the vasculature; however, the observed broad distribution of this isoform in both normal and neoplastic tissues makes it difficult to determine its exact role in carcinogenesis (Masferrer et al., 2000).

Several studies have indicated that COX-2 is upregulated, and consequently its prostaglandin products are more abundant, in several cancers including breast cancer, and that PGs appears to be important in cancer pathogenesis since they affect mutagenesis, mitogenesis, apoptosis, cellular adhesion, angiogenesis, and immune surveillance (Subbaramaiah et al., 1997; Parrett et al., 1997; Taketo, 1998; Hwang et al., 1998; Masferrer et al., 2000). COX-2 was reported to be upregulated in benign and malignant tumors but not in surrounding normal tissues (Eberhart et al., 1994; Kargman et al., 1995; Sano et al., 1995; Kutchera et al., 1996; Subbaramaiah et al., 1997). In addition, genetic evidence supporting a role for COX-2 in carcinogenesis has been reported, where intestinal tumorigenesis was observed to be inhibited in COX-2 knock out mice (DuBois et al., 1998). A null mutation for COX-2 caused a marked reduction in the number and size of intestinal polyps in murine model of familial adenomatous polyposis (the APC Δ716 knockout mice) (Oshima et al., 1996). Studies of colon cancer have also indicated that COX-2 upregulation occurs in colonic adenomas (Eberhart et al., 1994; Kargman et al., 1995), and that the increased levels of COX-2 in tumor tissue may reflect enzyme induction in both epithelial
and non epithelial inflammatory cells (Sano et al., 1995). Furthermore, experiments testing a selective COX-2 inhibitor on two different colon cancer cell lines, one of which has a high levels of COX-2 expression and activity, have reported that the selective inhibitor decreased cellular growth in both **in vivo** and **in vitro** assays only in the COX-2 expressing cell line (DuBois et al., 1998).

Elevated levels of prostaglandin E₂ (PGE₂) have been reported in malignant human breast tumors, and these high levels were often associated with tumors exhibiting high metastatic potential (Schrey et al., 1995). The **in vitro** studies with human breast tumor cells have demonstrated an increased PGs, particularly PGE₂, production from malignant breast tissues compared to normal or benign tissue, and have shown the transcription of COX-2 to be enhanced in transformed mammary epithelial cells (Schrey et al., 1995; Subbaramaiah et al., 1996). These observations, along with results from several studies in murine mammary tumors models, have suggested that PGs, particularly PGE₂, may indeed play a multi-functional role in controlling growth, metastasis, and host immune responses (Fulton et al., 1991; Subbaramaiah et al., 1996). Both the stromal and epithelial mammary cells have been implicated as potential sources of prostanoids, as studies in several breast cancer cell lines and breast fibroblasts, under the influence of inflammatory mediators, such as IL-1β and bradykinin (BK), as well as phorbol ester (TPA), have shown that breast fibroblasts represent a major source of PGE₂ production (Schrey et al., 1995). Although some experimental data have shown the PGE₂ production was not detected in hormone-dependent breast cancer cell lines, in contrast to the production of PGE₂ by hormone-independent cancer cell lines which exhibit an invasive phenotype, the rapid metabolic inactivation of
PGE₂ by the PG dehydrogenase in hormone-dependent cancer cells may have had limited its detection (Schrey et al., 1995).

Although the mechanisms of action of prostaglandins at the cellular level have yet to be elucidated, molecular studies have suggested that PGs interact through their specific receptors in the regulation of nucleic acid and protein synthesis (Lupulescu, 1996; Tjandrawinata et al., 1997; Wu et al., 2000), as well as cell communications (Schrey et al., 1995; Lupulescu, 1996). Thus, COX-catalyzed reactions may be important for carcinogenesis via several different mechanisms. For mutagenesis-related mechanisms, in addition to activating certain genes in the cytochrome P-450 family of enzymes, widely recognized for catalyzing oxidative reactions that convert xenobiotics (procarcinogens) to reactive electrophiles (proximate carcinogens/ mutagens) (Gonzalez et al., 1996), COXs can also convert a broad array of chemicals to mutagens during arachidonic acid metabolism including aromatic amines, benzidines, heterocyclic aromatic amines, and polycyclic aromatic hydrocarbons (PAHs) (Eling et al., 1992). Compounds like the PAHs can also transcriptionally induce COX-2 expression, thus enhancing their own metabolism (Kraemer et al., 1996; Kelley et al., 1997). The peroxidase activity contribution to the activation of these procarcinogens, which are associated with proteins and DNA adducts in such critical genes as the tumor suppressor gene p53 (Eling et al., 1990; Subbaramaiah et al., 1996), have suggested that increased levels of carcinogen-activating enzymes such as COX-2 may overwhelm the detoxification enzyme system, enhance cellular alterations, and favor tumor formation (Subbaramaiah et al., 1997). In addition, COXs may contribute to carcinogenesis through the metabolic by-products of arachidonate catalysis such as malondialdehyde.
(MDA), which is highly reactive and can form adducts with DNA, and was linked to mutagenesis in mammalian test systems (Smith et al., 1991; Marnett, 1992).

Tumor growth can be affected by either increased proliferation (mitosis) of cells or decreased rate of programmed cell death (apoptosis) (Shiff et al., 1996; Sheng & Shao et al., 1998). In regard to the mitogenesis-related mechanisms, the observations that prostanoids such as PGE$_2$, PGF$_{2\alpha}$, TxA$_2$ affect cell proliferation and alter the response of the immune system to malignant cells (Marnett, 1992; Muscat et al., 1995; Lupulescu, 1996; Shiff et al., 1996), have suggested that over expression of COXs (and overproduction of certain prostanoids) could favor malignant growth. Experimental studies have indicated that COX-2 is an immediate early gene associated with cell replication, and the increased synthesis of COX-2 derived products may allow increased proliferation of colonic epithelium early in the multistep carcinogenic process (Smith & DeWitt, 1996). The regulation of COX-2 by growth factors also suggests that COX-2, like other immediate early genes such as c-fos and c-myc, may play a role in mitogenesis, and perhaps a specialized role in wound healing (Smith et al., 2000). It was also proposed that prostanoids mediate the mitogenic responses to angiotensin II and EGF through stimulation of DNA synthesis (Makita et al., 1996). Further support comes from the observed NSAIDs inhibition in colon cancer of prostanoids production and the associated inhibition of tumor growth, thus preventing transition to malignancy (Muscat et al., 1995; Smith & DeWitt, 1996; Shiff et al., 1996). In addition, the reported contributions of COX-2 to the development of colon cancer may provide further support for a role for this enzyme in control of cell growth separate from that of COX-1 (DuBois et al., 1998). The PGE$_2$ has been reported to be mitogenic for normal human and
rodent mammary epithelial cells (Schrey, 1995), growth of osteoblast cells (Hughes-Fulford, 1992), and non transformed intestinal cell lines (Sheng et al., 1998). The growth-promoting effects of prolonged treatment with PGE₂ on the gastric epithelium have also been reported, and there is evidence for an interaction between PGs and growth factors expression in some tissues, where PGE₂ for example was reported to induce the hepatocyte growth factor (HGF) expression in skin and gastric fibroblasts (Jones et al., 1999). In addition, both the nonselective NSAID, indomethacin and NS-398 (a selective COX-2 inhibitor) were reported to suppress in vitro the proliferation of gastric cancer cells that overexpress COX-2, while exerting minimal effects on proliferation of other cancer cell lines which express lower levels of COX-2 (Sawaoka et al., 1998).

Of particular importance to mammary tumor growth may be the indirect role of COX and PGs. The increased production of PGE₂ may have a paracrine effect on neighboring cells, in which it may activate the P-450 CYP19 aromatase gene which leads to stimulation of estrogen biosynthesis, and estrogen has been associated with cellular proliferation (Simpson et al., 1994; Zhao et al., 1996; Russo et al., 2001). In addition, several human breast cancer cell lines have been observed to produce endothelin-1 (ET-1) which may exercise a paracrine mitogenic influence on neighboring cells that may partially account for the extensive desmoplasia seen in breast tumors, and PGE₂ was shown to stimulate the production of ET-1 in breast cancer cells (Patel et al., 1996). On the other hand, ET-1 was shown to stimulate PGE₂ production in breast stromal fibroblasts, suggesting a bidirectional paracrine crosstalk between epithelial and stromal cells (Schrey et al., 1995). It was also shown that COX-2 was induced by estrogen in ovarian granulosa cells (Wu, 1996).

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On the mechanistic aspect, however, the precise role of PGs in proliferation of cancer cells is not well clarified, and the controversy over interpretation of the observed associations between different prostanoids and cellular proliferation remains to be settled, and the exact molecular mechanisms underlying such PGs mitogenic/anti mitogenic effects are yet to be elucidated. For example, it is not clear if the growth-stimulatory effects of certain PGs involve an increase in the rate of cell proliferation (cell cycle progression) or a decrease in the rate of cell death (apoptosis). Related to the uncertainty are the proposals that PGs may probably play different roles in normal (physiological) as compared to abnormal (pathological) conditions such as those seen in cancers; and that some prostaglandins such as PGA and PGJ (dehydration products of PGE), which have been reported to exhibit anti-proliferative effects, could be derived from other prostaglandins. Thus, depending on the type of cells and the particular conditions of each experimental model (e.g. the medium or the inducer used), the reported results are mixed. Some experimental data have suggested that PGE$_2$ may inhibit growth of human breast cancer cells, and DNA synthesis in human breast fibroblasts (Schrey et al., 1995), however, the possibility of spontaneous or protein-catalyzed dehydration of PGE$_2$ to PGA$_2$ (a cyclopentenone PG, and like PGD and PGJ are proposed to be growth inhibitory) cannot be ruled out (Marnett, 1992; Fukushima et al., 1992; Taketo, 1998). Still, one study has reported that PGJ$_2$ has induced proliferation of colorectal cancer cells (Chinery et al., 1999).

In contrast to these reports, several PGs, including PGE$_2$, were observed to stimulate proliferation and DNA synthesis in rat mammary tumor cells (Lee et al., 1992); and PGE$_2$ was reported to enhance proliferation and increase clonogenicity of colon cancer cell lines.
(and decrease apoptosis); and to significantly increase cellular proliferation and total DNA content in human prostatic carcinoma cells (Tjandrawinata *et al*., 1997; Sheng & Shao *et al*., 1998). In addition, NSAIDs were reported to inhibit proliferation, through effects on cell cycle, of colon adenocarcinoma cells, and induce apoptosis (Shiff *et al*., 1996). Also, in esophageal cancer cells producing large amounts of PGE₂, COX-2 inhibitors were reported to reduce the proliferative activity (and induce apoptosis) through the inhibition of PG synthesis (Zimmermann *et al*., 1999). In addition, some experimental data have suggested that PGE₂ and PGF₂α enhance proliferation of epidermal carcinomas, presumably through increasing DNA synthesis and modulating cAMP levels (Lupulescu, 1996). Another prostanoid, TxA₂, was reported to stimulate proliferation of rat mammary cancer cell lines, and *in vivo* animal models, and the analyses of plasma levels of TxA₂ metabolites in breast cancer patients have indicated a dramatic decrease following removal of the tumors and an equally dramatic increase coincident with tumor recurrence (Marnett, 1992). Studies of the effects of PGs on vascular smooth muscle cells (VSMC) have shown the vasodilatory PGs such as PGI₂ and PGD₂ to decrease the proliferation of VSMCs, whereas the vasoconstrictor TxA₂ to enhance proliferation of these cells; and the rapidly growing and infiltrating brain tumor glioma was reported to have higher synthesis of TxA₂, whereas synthesis of PGD₂ and PGI₂ are diminished (Fukushima *et al*., 1992).

Several studies have suggested COX-dependent *apoptosis-related* mechanisms. It seems that the apoptotic effects of increased COX-2 expression or PGE₂ treatment depend on the particular cell type being evaluated. Several reports have indicated that such treatment leads to inhibition of apoptosis in cell culture systems including those of epithelial origin,
however, treatment with PGE$_2$ was reported to induce B cells to undergo apoptosis (Brown et al., 1996; DuBois et al., 1998). Over expression of COX-2 in epithelial cells was reported to make them resistant to apoptosis, increase their tumorigenic potential, and increase the invasiveness of tumor cells (Subbaramaiah et al., 1997; DuBois et al., 1998). Intestinal epithelial cells over expressing COX-2 were shown to have enhanced expression of the anti-apoptotic protein Bcl-2, reduced TGF-β$_2$ receptor levels, and to be resistant to undergoing apoptosis, as well as to have enhanced ability to bind to extracellular matrix; and treatment with the NSAID, sulindac sulfide reversed the resistance to apoptosis induced by COX-2 overexpression (Tsujii et al., 1995). Treatment of human colon cancer cells with PGE$_2$ was reported to inhibit apoptosis, induce MAPK kinase activity, and induce Bcl-2 expression (Sheng & Shao et al., 1998). By inhibition of apoptosis, the increased synthesis of COX-2 products may allow continued survival of epithelial cells early in the multistep carcinogenic process (Tsujii et al., 1995) which can enhance the tumorigenic potential of these cells.

There are several malignant transformation-related phenotypes reported to be associated with COXs. In mammary epithelial cells stimulated by cytokines or growth factors, the upregulation of COX-2 was reported to be associated with cellular transformation, and in vitro studies of mammary epithelial cells have shown that COX-2 transcription and PGE$_2$ production were markedly increased in transformed cells compared to the non transformed cells, or normal cells (Subbaramaiah et al., 1996). Colon cancer cells permanently transfected with COX-2 were shown to acquire increased invasiveness compared with control or parent cells, suggesting that over expression of COX-2 in a cancer cell lines may lead to phenotypic changes associated with increased metastatic potential; and
activation of metalloproteinases was a reported change associated with the increased invasiveness (Tsujii et al., 1995; 1997). The increased production of PGs and invasiveness were observed to be reversed by treatment with the NSAID, sulindac sulfide (Tsujii et al., 1997). Over expression of COX-1 and -2 in human breast cancer cells was also shown to mediate the activation of metalloproteinases involved in cellular motility (Takahashi et al., 1999). Intestinal epithelial cells overexpressing COX-2 were shown to exhibit decreased expression of the receptor for the (growth inhibitory) transforming growth factor β2 (TGF-β2); as well as E-cadherin involved in cell-cell adhesion (Tsujii et al., 1995). COX-2 upregulation (and increased PGE2 production) in rat fibroblasts was shown to be induced by activated ras oncogene which is reported to be associated with cancer transformation and progression (Sheng et al., 1998); and the upregulation of COX-2 is a downstream effect of ras-mediated transformation in mammary epithelial cells (Subbaramaiah et al., 1996). All are changes that enhance the tumorigenic potential of epithelial cells. The COX-2 derived PGs were also reported to mediate tumor growth and metastasis in two independent colon and lung animal models (Masferrer et al., 2000).

Drugs that modify PGs synthesis have been shown to affect the numbers of both experimental and spontaneous metastases formed in experimental animals, probably by affecting platelet aggregation (Hill et al., 1992). Inflammation, particularly chronic inflammation, is a recognized risk factor for several types of epithelial cancer, including breast cancer (Subbaramaiah et al., 1997; Prescott et al., 2000). Inflammation was observed to increases the synthesis of PGs, in part through the upregulation of COX-2, and breast cancer tissues were shown to exhibit similarity with the processes involved in inflammation,
connective tissue remodeling and wound repair, including infiltration of cytokine-producing host immune cells (Schrey et al., 1995). In addition, prostaglandins have been implicated in the mastalgia (benign breast disorder) associated with premenstrual syndrome (Schrey et al., 1995). Also inflammatory mediators were shown to activate phospholipase A₂, and the released fatty acids, aside from being substrates for COXs, can act as second messengers affecting cellular signaling pathways such as PKC (Blobel et al., 1995). Studies of membrane-associated phospholipase A₂ in breast tissue have reported its high expression in malignant versus benign or normal tissue (Yamashita et al., 1993). The overexpression of group II phospholipase A₂ (and subsequent increase in fatty acids and PG production) in human breast cancer tissue was also reported to be associated with malignant potency (Yamashita et al., 1994). The fibroblastic inhibition of osteoblasts functional differentiation (inhibition of calcification through inhibiting alkaline phosphatase generation and activity) was shown to be mediated in part by the release of PGE₂ and PGF₂α, whereas PGD₂ and its metabolite PGJ₂, have been shown to have marked stimulatory effects (vitamin D-like stimulation of alkaline phosphatase generation and activity-dependent calcification) on osteoblast differentiation (Fukushima et al., 1992).

The growth of various tumors is often associated with immune suppression (Subbaramaiah et al., 1997), and the observations that COXs-synthesized prostaglandins have demonstrated immunosuppressive effects in the form of impairing the immune surveillance and killing of malignant cells (Subbaramaiah et al., 1996; 1997) make it possible to assume that upregulation of COX-2 prolongs the survival of abnormal cells, which favors the accumulation of more alterations and increases the risk of carcinogenesis.
Growth factors released by tumor cells may activate monocytes and macrophages to synthesize PGE₂, which was observed to inhibit monocyte functions (Zeidler et al., 2000), to modulate the production of immune regulatory cytokines (Fedyk et al., 1997), and to inhibit lymphocytes' proliferation, and the cytotoxic activity of the natural killer lymphocyte cells (Lupulescu, 1996; Subbaramaiah et al., 1997). Also PGE₂ was reported to inhibit the production of macrophage tumor necrosis factor (TNF), while inducing the production of the cytokine IL-10 which has immunosuppressive effects (Kambayashi et al., 1995; Fedyk et al., 1997). Also, PGI₂ was reported to block leukocyte adhesion to endothelial cells and to inhibit monocyte activation (Wu, 1996). In addition, treatment with inhibitors of COX-2 including NSAIDs have been reported to attenuate the tumor-mediated immune suppression (Huang et al., 1994; Castonguay et al., 1997).

Although the expression of COX-2 is observed to be associated with malignant transformation, the precise role of COX-2 in transformation is not clear. Similar to the uncertainties related to prostanoid roles in proliferation, some published data on COX-2 roles in transformation suggest that increased COX-2 activity is not sufficient for transformation of rat intestinal epithelium, and selective COX-2 inhibitors did not reverse the transformed phenotype caused by ras in COX-2 expressing colon cancer cell lines (Sheng et al., 1998). However, the forced expression of COX-2 in rat intestinal epithelial cells has resulted in resistance to apoptosis (Sheng & Shao, 1998), and selective inhibition of COX-2 activity in ras transformed intestinal cells and colon cancer cell lines, was shown to inhibit the growth of these cells, primarily via the induction of apoptosis, and to decrease the tumorigenicity of the transformed cells (Sheng & Shao, 1998). These apoptosis-related effects further support
the proposal that increased COX-2 and PGs production that occur during cell transformation may enhance the cell survival and provide a selective advantage for the transformed cells that overexpress COX-2.

COXs were also implicated in angiogenesis-related mechanisms. Recent reports have indicated that COXs may play a vital role in the regulation of angiogenesis associated with neoplastic tumor cells (Tsujii et al., 1998, DuBois et al., 1998; Nishimura et al., 1999; Masferrer et al., 2000). The PGE1 and PGE2, have been implicated as positive (angiogenic) regulators in angiogenesis (Graeber et al., 1990). In breast tissues, in addition to PGE1 and PGE2, nitric oxide, as well as the arachidonic acid product 12 R-HETE have also been reported as non-peptide angiogenic factors involved in angiogenesis (Graeber et al., 1990; Leibovich et al., 1994; Laniado et al., 1994; Weidner et al., 1996). Furthermore, when endothelial cells were stimulated with the 12 R-HETE, the proto-oncogenes c-myc, c-jun, and c-fos were activated (Laniado et al., 1994). COXs were proposed to regulate cancer-induced angiogenesis by two mechanisms: COX-2 can modulate production of angiogenic factors by cancer cells, while COX-1 can regulate angiogenesis in endothelial cells (Tsujii et al., 1998). It was reported that COX-2 overexpressing colon carcinoma cells, co-cultured in vitro with endothelial cells, produce high levels of PGs and pro-angiogenic factors and stimulate both endothelial migration and tube formation; and these effects were inhibited by a COX-2 inhibitor and by aspirin. However, treatment of endothelial cells with COX-1 antisense oligonucleotide or aspirin was observed to inhibit COX-1 and suppress tube formation (Tsujii et al., 1998). In contrast, in an in vivo FGF-2 induced angiogenesis model, endothelial cells present in the established vessels were reported to express COX-1 whereas
newly vascularized endothelium expressed COX-2; and selective COX-2 inhibition (but not COX-1) was shown to produce anti-angiogenic effect in this rat model of angiogenesis (Masferrer et al., 2000).

1.5. Chemoprevention.

Several lines of evidence have suggested that a large fraction of human cancers may be potentially preventable, since many of the associated factors that determine its incidence are largely exogenous in nature (Weinstein et al., 1995). Cancer chemoprevention can be generally defined as prevention of cancer occurrence, especially in high risk populations, through the regular and prolonged intake of chemical compounds, either naturally occurring constituents of the diet or synthetic (Morse et al., 1993). There is a number of naturally occurring substances and several synthetic compounds that were reported to inhibit cancer induction in experimental animals (Weinstein, 1991; Stoner et al., 1991; Sugimura et al., 1992; Chen et al., 1993; Wattenberg et al., 1993), however, the precise roles of these agents in cancer prevention are not well elucidated. Epidemiological and experimental studies have suggested that agents such as retinoids are useful in cancer prevention, and a diet rich in vegetables decrease the risk of certain cancers (Weinstein, 1991; Hong et al., 1990). Also, in view of the association of breast and colon cancer with a high-fat diet, it seems likely that the incidence of these diseases can be reduced by decreasing the consumption of certain types of fat (Weinstein et al., 1995, Rose et al., 1997).

The anticarcinogenic agents appear to act at various stages of the carcinogenic process and by various mechanisms. The traditional classification of these chemopreventive
substances as blocking (anti-initiating) or suppressing (anti-promoting/anti-progressing) agents was essentially based on the classical chemically-induced initiation/promotion model of carcinogenesis. However, such classification may face difficulties similar to those encountered in the attempts to classify initiators and promoters, that is, the sequence of events and the precise roles or mechanisms played by the putative carcinogens are poorly understood. Therefore, a more flexible classification could be mechanistic-based, so that chemopreventive agents could be classified as: {1} agents that reduce the absorption of carcinogens, such as dietary fibers; {2} agents that reduce the synthesis of carcinogens in the body, such as isothiocyanates or ellagic acid (act as inhibitors of cytochrome P450), caffeic acid, and ferulic acid, which inhibit the formation of nitrosamine; {3} agents that inhibit (scavenging electrophiles) the covalent binding of carcinogenic agents to DNA and proteins, such as elagic acid and flavonoids (present in fruits and vegetables); {4} agents that alter the metabolism of carcinogens and can act as antioxidants that inhibit oxidative damages, such as benzyl isothiocyanate (present in cruciferous vegetables like cabbage), ascorbic acid, selenium and β-carotene; {5} agents that inhibit tumor transformation and act as inducers of differentiation, such as retinoids, β-carotene, α-tocopherol, and calcium (all present in fruits and vegetables); and {6} agents that inhibit tumor formation by various mechanisms (sometimes not yet established) such as organosulfur compounds (in garlic and onions); NSAIDs and curcumin (in turmeric/curry) which act as inhibitors of arachidonic acid metabolism (curcumin also inhibit PKC pathway and AP-1 mediated gene expression); capsaicin (in chili peppers); polyphenols (in green tea) which act as inducers of phase II enzymes; and various protease inhibitors. Some agents like limonene may act by inhibiting...
post translational modification of the p21WAF1 protein by a farnesyl residue, thus providing a strategy to interfere with the action of an activated oncogene (Huang et al., 1991; Crowell et al., 1991; Morse et al., 1993; Weinstein et al., 1995).

1.6. NSAIDs: Description and Classification.

The anti-inflammatory effects of the nonsteroidal anti-inflammatory agents (NSAIDs) appear to stem predominantly from blockade of the prostaglandin synthesis cascade by inhibition of its rate limiting isozymes, COX-1 and -2 (Herschman et al., 1994). Both COX-1 and -2 are important pharmacologically as major therapeutic targets of NSAIDs. For example, COX-1 may be considered a relevant target for NSAIDs such as low doses of aspirin (known to influence hemostasis) acting to inhibit platelets’ TXA₂ formation (Willard et al., 1992; Patrignani et al., 1994), whereas COX-2 may be considered a relevant target of selective NSAIDs acting to inhibit the development of inflammation, pain, fever (Seibert et al., 1994; Futaki et al., 1994; Chan CC et al., 1995), cancer (Eberhart et al., 1994; Giovannucci et al., 1995; Kutchera et al., 1996), and perhaps Alzheimer disease (Schnabel et al., 1993; Vane et al., 1998).

Clinically, there are two classes of drugs that affect prostanoid metabolisms and therefore are therapeutically useful. The first are the NSAIDs which are subdivided further into nonselective agents such as aspirin (acetylsalicylic acid), ibuprofen, indomethacin, peroxicam, sulindac sulfide and phenylbutazone; and COX-2 selective inhibitory agents such as celecoxib and refecoxib, which block prostanoids biosynthesis by inhibiting COX-2 isozyme. The second group, the steroids’ anti-inflammatory agents, such as hydrocortisone,
prednisone, betamethasone and dexamethasone, appear to act both by inhibiting the phospholipases A₂ so as to interfere with mobilization of arachidonic acid, and by inhibiting COX-2, thus blocking the release of prostaglandins (Glew, 1992; Smith & DeWitt, 1996; Smith et al., 2000).

NSAIDs that inhibit cyclooxygenase catalytic activity, could be conveniently classified into three classes according to their differences in the manner with which they interact with the cyclooxygenase active site (Smith & DeWitt, 1996; Taketo, 1998). In other words, on the basis of their binding kinetics with the COXs they can be grouped according to the following equation: \[ E + I \rightarrow EI = EI^* \].

The class I inhibitors, which include ibuprofen, mefenamic acid, flufenamic acid, peroxicam, sulindac sulfide, naproxen, and 6-methoxy-naphthyl-2-acetic acid, are simple competitive cyclooxygenase inhibitors that rapidly but reversibly form an enzyme-inhibitor (EI) complex only (Smith & DeWitt, 1996; Taketo, 1998). The class II inhibitors, which include indomethacin, meclofenamic acid, flurbiprofen, and diclofenac, are competitive, time-dependent, reversible inhibitors. They form an EI complex, then change the structure of the protein to produce an EI* complex, but without covalently modifying the protein. The EI* complex formation is relatively slow (seconds to minutes), and the EI* slowly revert to EI (Laneuville et al., 1994). The mechanistic basis for the ability of a class II NSAID to cause the formation of semi-stable EI* complex is not well understood. The COX-2 selective agents are considered non covalent, time-dependent, reversible inhibitors of COX-2 (Smith & DeWitt, 1996; Taketo, 1998). The physical basis for time-dependent inhibition is not well defined, and may be different for different agents (Smith et al., 2000). However, one
explanation proposed for the selectivity of COX-2 inhibitors is that they inhibit COX-2 isoform by a time-dependent, pseudo irreversible mechanism, whereas they inhibit COX-1 by a rapid, competitive, and reversible mechanism; and the practical result of this mixed mode of inhibition is that COX-1 will be minimally affected while COX-2 will be effectively inactivated when the blood concentration of the COX-2 inhibitor is below that required for half-maximum inhibition (IC$_{50}$) of COX-1 (Smith et al., 2000).

The *class III* inhibitors, which include aspirin, propinonylsalicylate, and valerlsalicylate, convert El to an El* complex by covalent modification (acylation) of the protein. Once the El* complex is formed with an inhibitor of this class, the protein does not revert back to El, and the cell must synthesize new COXs to regain cyclooxygenase activity (Bhattacharyya et al., 1995; Taketo, 1998). Aspirin binds to the cyclooxygenase active site with a very low affinity, however, it transfer its acetyl group to a specific active site residue (Mancini et al., 1994; Lol et al., 1995) to covalently modify the COXs. The effect of aspirin on COX-1 vs. COX-2 activity is somewhat different, as the acetylation of COX-1 completely inhibits the cyclooxygenase activity (but does not affect the peroxidase activity), while acetylation of COX-2 by aspirin convert this enzyme to a form that still oxygenate arachidonic acid to yield 15R-hydroxyeicosatetraenoic acid (15R-HETE) instead of PGG$_2$ (O'Neill et al., 1994; Taketo, 1998). Various other acyl salicylates such as valerylsalicylate and propinonylsalicylate have been reported to be cyclooxygenase activity inhibitors, where valery(pentanoyl)salicylate was reported as a relatively selective inhibitor of COX-1, whereas the propinonylsalicylate was observed to be about equally effective against COX-1 and COX-2 (Bhattacharyya et al., 1995).
The kinetic differences of NSAIDs inhibition have made comparisons of the agents interactions with COX-1 vs. COX-2 difficult, particularly in vivo (Smith et al., 2000). Nevertheless, values for the instantaneous inhibition of cyclooxygenase activity of human COX-1 and -2 by nonselective NSAIDs have been reported (Smith & DeWitt, 1996). In general, the common nonselective NSAIDs have higher relative affinities for COX-1 than COX-2. For example, ibuprofen IC\textsubscript{50} (in uM) for hCOX-1 is 4.0 ± 1.0 (SEM) and for hCOX-2 is 12.5 ± 2.1 (Laneuville et al., 1994), which is consistent with determinations of substrate specificity that suggest the cyclooxygenase active site of COX-2 to be somewhat larger and more accommodating than that of COX-1 (Smith & DeWitt, 1996).

The efforts to develop COX-2 inhibitors were driven initially by the notions that COX-2 is the relevant enzyme in inflammation (Masferrer et al., 1994; Seibert et al., 1994; Smith et al., 2000), that COX-1 is predominantly present in the stomach (Smith et al., 2000), and by the structurally different NSAIDs' binding sites of COX-1 and -2 (Futaki et al., 1994; Subbaramaiah et al., 1997). The COX-2 selective inhibitors are observed to exhibit relatively poor inhibition of COX-1, probably due to amino acids difference (within the hydrophobic cyclooxygenase catalytic channel) between COX-1 and COX-2 (Gierse et al., 1996; Smith & Garavito, 1996).

**Adverse Side-Effects:** NSAIDs in general have exhibited several adverse side effects associated with high dosage and chronic usage, including those in the gastrointestinal (GI) tract such as peptic ulcer disease and GI bleeding (serious adverse GI effects were reported to occur at any time and may not be preceded by warning symptoms or signs), renal (in susceptible individuals), hematologic such as bleeding and aplastic anemia (which was
reported to result from phenylbutazone therapy); hepatic, nervous system, otic, ocular, cartilage destruction, and dermatologic adverse effects. The GI, renal, and hematologic toxicities are considered the most important adverse effects (Soll et al., 1991; Smith et al., 1995; Rainsford, 1999; McEvoy et al., 2000). The commonly used nonspecific NSAIDs appear to inhibit both COX-1 and COX-2 isozymes which probably account for both their therapeutic and adverse side effects such as gastrointestinal and renal toxicities. The NSAIDs-mediated inhibition of COX-1 appears to abrogate the presumed housekeeping functions of this isoform such as cytoprotection of gastric mucosa, regulation of renal blood flow, and control of platelet aggregation, thereby causing such side effects. To date, the reported evidence of COX-2' physiological roles in various tissues makes it possible to consider that selective COX-2 inhibition might be associated with fewer adverse effects than are seen with the standard nonselective NSAIDs. The recent development and release of selective COX-2 inhibitors such as celecoxib and refecoxib have, therefore, attracted considerable attention because of their intended ability to selectively inhibit the inducible COX-2 isoform while sparing COX-1, thus may reduce the gastric side effects which have limited the wide use of NSAIDs as chemopreventive agents. For example, endoscopic studies have shown that selective COX-2 inhibitors have caused much less injuries to the mucosa of the upper gastrointestinal tract than nonselective NSAIDs (Bjarnason et al., 1997).
CHAPTER 2

MATERIAL AND METHODS

The animal model of mammary carcinogenesis used in this preclinical efficacy research is the chemically (DMBA)-induced rat mammary cancer model. This established animal model is known to adequately demonstrate several general principles in cancer causation development and growth, in addition to its relevance to human breast cancer in various aspects. The complete carcinogen, the polycyclic aromatic hydrocarbon (PAH), DMBA (7,12-dimethylbenz(a) anthracene) was used as the initiating carcinogen in Sprague Dawley rats.

2.1 Reagent and Chemicals.

2.1.1. DMBA(7, 12-dimethylbenz(a)anthracene). Chemically, DMBA is a procarcinogen that is not highly reactive, and it undergoes metabolic activation in vivo to highly reactive electrophilic species that can react with nucleophilic residues in cellular proteins and nucleic acids to form covalent adducts (Cooper et al., 1982). Several diols and diol-epoxides are produced in the metabolic activation of DMBA in rat mammary gland including DMBA-3,4-diol, DMBA-5,6-diol, DMBA-8,9-diol, and DMBA-10,11-diol; and the DMBA-3,4-diol-1,2-
oxide, and DMBA-10,11-diol 8,9-oxide (Cooper et al., 1982). The cytochrome P-450 oxidative system in the endoplasmic reticulum (ERet) has been reported to predominantly convert DMBA to such variety of derivatives. Among the normal roles of this system (Phase-I enzymes) is to convert lipid-soluble foreign substances to more water-soluble substances that can be excreted. However, some of the intermediates in this oxidative process are epoxides, which can form covalent bonds with proteins and bases in DNA and RNA. The DMBA diol epoxides were reported to form covalent adducts with primarily the adenine residues in DNA (Cooper et al., 1982; Cheng et al., 1988), and the probable adduct to be N^6 dA-DMBA (Cardiff et al., 1988; Brown et al., 1990). The DMBA modification of DNA can result in distortions in the conformation of the DNA helix, which in turn can result in base displacement and a variety of mutations (Archer, 1992; Weinstein et al., 1995). Although DMBA reactive species can attack different sites on the DNA molecules and produce different distortions in the structure of DNA, the major DNA adducts are often the same in different species and tissues, and the reported type of mutation often associated with DMBA is the A→T transversion (Weinstein et al., 1995).

A series of enzymes (Phase-II enzymes) including epoxide hydrolase, glutathione-S-transferase, sulfotransferase, UDP-glucuronyl transferase, and NADPH quinine reductase have been reported to be involved in further metabolism and detoxification of activated carcinogens and other xenobiotics, thus preventing their binding to DNA (Guengerich et al., 1993; Nebert et al., 1991). Polymorphism in both phase I and II enzyme systems is considered an important variable in animals and human susceptibility to chemical carcinogenesis (Rothman et al., 2001).
It has reported that the majority of DMBA-induced rat mammary tumors contain a mutationally activated ras gene (Barbacid, 1987; Brown et al., 1990). It is worthy to note that unlike experimental rodents mammary tumors where mutational activation of ras family of proto-oncogenes was observed to be common, in human breast cancer where over expression of ras was observed in the majority of cases, mutations are seldom seen (Dickson et al., 1995; Miller et al., 1996). Interestingly, the p53 suppressor gene mutations observed in human breast cancer have been reported to be associated with the mutational spectrum (which differs from that of DMBA) of reactive oxygen species such as 8-OH-dG (Prosser et al., 1990; Yang et al., 1991; Coles et al., 1992). The 8-OH-dG induced lesions were observed to be primarily G→T transversion, the type of base substitution observed in the p53 gene from breast tumors (Cheng et al., 1992). Thus, although the factors that cause p53 deactivation in breast cancer are not fully known, it could reflect oxidative damage that may have led to formation of DNA adducts. Oxidized bases have been detected in breast tissue, and the concentration of total modified bases in tumor samples was reported to be 9-folds higher as compared with normal control values (Malins et al., 1991).

The capacity of the rat mammary gland to activate DMBA to carcinogenic forms has been demonstrated in vitro, in vivo, and in situ (Christou et al., 1987). Although DMBA-induced mammary carcinomas arise predominantly from epithelial cells, the two major cell populations of the virgin rat mammary gland, the parenchymal (epithelial cells) and the stromal (mainly fibroblasts) cells are both able to metabolize DMBA to a variety of mono-oxygenated derivatives (Christou et al., 1987; Gould et al., 1982). Interestingly, the weak mammary carcinogen benzo(a)pyrene (BP) was reported to be activated by the stromal cells.
and not by the parenchymal cells from which mammary carcinomas arise (Gould et al., 1982). The stromal cell population was reported to metabolize DMBA at approximately the same rate as the epithelial cells, however, epithelial cells generally maintained higher intracellular concentration of DMBA compared to those of the stromal cell population (Moore et al., 1983). The DMBA for our experiments was obtained as powder for oral administration (Sigma Chemical Co., St. Louis, MO).

2.1.2. The Nonselective NSAID, Ibuprofen. Ibuprofen is a prototypical nonsteroidal anti-inflammatory agent. Its solubility is very poor in water and very soluble in alcohol. It has shown anti-inflammatory, antipyretic, and analgesic activities in both humans and animals. The exact mechanisms of action of ibuprofen have not been clearly established, but many of the actions appears to be associated principally with the inhibition of both COX isozymes and thereby of PGs synthesis (McEvoy et al., I, 2000). COX-1 inhibition is presumably responsible for the drug's unwanted side effects on GI mucosa and platelet aggregation (McEvoy et al., I, 2000). Approximately 80% of an oral dose of ibuprofen is absorbed from the GI tract. Absorption is slower and plasma concentration are reduced when taken with food, however, the extent of absorption is not affected (McEvoy et al., I, 2000). Following an oral dose of 200 mg ibuprofen oral suspension in adults, the peak plasma concentration reported was 20 μg/ml, reached in about 47 minutes. It was slightly higher in children. The plasma half-life of the drug has been reported to be 2-4 hours, and elimination is essentially complete within 24 hours (McEvoy et al., I, 2000). The plasma concentration required for the anti-inflammatory effects are not known, and it has been reported that up to two weeks of therapy may be required before therapeutic response occurs. The usual doses for
inflammatory diseases are 400-800 mg 3–4 times/day (McEvoy et al., II, 2000). Studies of the pharmacokinetics of Ibuprofen have shown that absorption, distribution, metabolism and clearance are mechanistically similar in animals and humans (Kelloff et al., 1994; Shah et al., 1987; Davis et al., 1994). Ibuprofen for our experiments was obtained as Motrin (100mg/5ml) oral suspension (McNeill Pharmaceuticals, Fort Washington, PA).

2.1.3. The Selective COX-2 inhibitor NSAID, Celecoxib. Molecular-based targeting for the inhibition of the COX-2 isoform have led to the development of a new class of NSAIDs, the selective COX-2 inhibitor such as celecoxib, that potently inhibit COX-2. Celecoxib has been reported to have >300 fold selectivity in vitro for COX-2 versus COX-1 (Masferrer et al., 2000). These agents have significant anti-inflammatory, antipyretic and analgesic properties while avoiding typical side effects associated with the nonspecific NSAIDs such as aspirin and indomethacin which inhibit both COX-1 and COX-2 (Penning et al., 1997). The IC₅₀ of celecoxib for human COX-1 = 15 uM, and for human COX-2 = 0.04 uM (Tally et al., 2000). The drug solubility is poor in aqueous solution with pH less than 9, and increases in strong basic solutions. It is highly soluble in alcohol (McEvoy et al., II, 2000). Although the exact mechanisms of action are not clearly established, celecoxib, like other selective NSAIDs is assumed to act through selective inhibition of COX-2 isozyme and thereby reduces the production of prostaglandins. At clinically relevant concentrations, celecoxib was shown to inhibit COX-2 in a slow time-dependent manner, that involve the formation of a tight enzyme-drug complex that is non covalent but only slowly dissociable (DeWitt, 1999). Celecoxib has been evaluated in patients with familial adenomatous polyposis (FAP), and showed a 28% reduction in polyps numbers with 400 mg twice daily.
(McEvoy et al., II, 2000). The efficacy of an NSAID as a chemopreventive/therapeutic agent is likely to depend on its tissue availability (local bioavailability), and studies of celecoxib's tissue distribution have shown that the drug is extensively distributed in body tissues (McEvoy et al., II, 2000). Celecoxib is well absorbed from GI tract, and peak plasma concentration of 705 ng/ml is attained in less than three hours after a single dose of 200 mg, and the effective half-life is about 11 hours. The peak plasma concentration is about 40% higher in black than white, although the causes are not known. The bioavailability of the drug was reported to increased when concomitantly administered with high fat meal. The steady state plasma concentration are reached within 5 days, and no drug accumulation was observed in individuals receiving 400 mg twice daily (McEvoy et al., II, 2000). The drug is metabolized in the liver to inactive metabolites principally by the cytochrome P-450 (CYP) isozyme 2C9, and drugs that inhibit CYP2C9, such as flufastatin and fluconazole, may affect the pharmacokinetics of celecoxib. Also celecoxib was shown to inhibit CYP2D6, and the possibility exists that celecoxib may alter the pharmacokinetics of drugs metabolized by this enzyme such as some β-blockers, and antidepressants (McEvoy, II, 2000). Although the drug have been associated with a lower incidence of adverse upper GI effects, e.g. very low ulcerogenic effects in humans, animal studies have shown that COX-2 may contribute to GI ulcers healing and inhibition of COX-2 may interfere with that healing process (McEvoy et al., II, 2000). Unlike prototypical NSAIDs, celecoxib does not appear to inhibit platelet aggregation. COX-2 inhibitors are recently introduced agents in clinical settings, and although celecoxib has been reported to be associated with very low incidence of adverse renal effects, it has been associated, like prototypical NSAIDs, with mild adverse effects such
as edema, albuminuria, and increased BUN (blood urea nitrogen) and serum creatinine concentration (DeWitt, 1999). There is also some evidence that administration of NSAIDs including celecoxib may reduce the blood pressure response to ACE (angiotensin converting enzyme) inhibitors (McEvoy et al., II, 2000). Celecoxib (SC-58635: 4-[5-(4-methyl phenyl)-3-(trifluoromethyl)-1H- pyrazol-1-yl]benzene-sulfonamide was obtained for our experiments as powder for oral administration (Searle-Monsanto Research and Development, St. Louis, MO).

2.1.4. The Selective COX-1 inhibitor NSAID, SC-560. SC560 is an experimental highly selective agent that inhibit the constitutively expressed COX-1 isozyme. Its water solubility is poor, and alcohol solubility is very high. The IC_{50} of SC560 for animal COX-1 = 0.009 uM (human COX-1 = 0.007), and for animal COX-2 = 6.3 uM (human COX-2 = 7.5)(Smith CJ et al., 1998). Given orally to experimental animals, it was shown to inhibit COX-1-derived TxB_2, gastric PGE_2, and dermal PGE_2 production, but did not inhibit COX-2-derived PGs in lipopolysaccharide-induced rat inflammatory model (Masferrer et al., 2000). The human toxicity evaluation for this agent has not been reported. SC560 was obtained for our experiments as powder for oral administration (Searle-Monsanto Research and Development, St. Louis, MO).

2.1.5. The retinoid, 4-HPR. Retinoids, both naturally occurring and synthetic analogues such as 4-HPR (4-hydroxyphenyl retinamide) were reported to suppress carcinogenesis in various tissues by stimulating differentiation, apoptosis, and immune recognition of aberrant cells (Subbaramaiah et al., 1997). In addition to their established role as differentiating agents, retinoids have been shown to down regulate COX-2 (Subbaramaiah et al., 1997), to
inhibit the metabolism of arachidonic acid (El-Attar et al., 1991), to suppress phorbol ester-mediated induction of COX-2 in human oral epithelial cells (Mestre et al., 1997), and to antagonize AP-1 mediated transcription (Schule et al., 1991). 4-HPR was obtained for our experiments as powder for oral administration (McNeil Pharmaceuticals, Springhouse, PA).

2.1.6. The experimental animals and Diet. The DMBA-induced mammary tumorigenesis model in Sprague Dawley rats is a well established mammary cancer model. The female virgin Sprague Dawley (SD) are outbred albino rats, with very high susceptibility to develop mammary tumors (essentially 100% when given a carcinogen at age range 35-55 days (Russo & Russo II, 1997). The animals were purchased from Harlan Industries, Indianapolis, IN. The Teklad chow 22/5 rodent diet (w):8640 is a complete diet for rats, which contains 22% crude protein, 5% crude fat, and 4.5% crude fiber. Teklad chow 22/5 rodent diet (w):8640 was purchased from Harlan Industries.

2.1.7. Other reagents and Chemicals. 5-Bromo-2'-deoxyUridine (BrdU) and Phosphate Buffered Saline (PBS) were purchased from Sigma Chemical Co., St. Louis, MO. The tissue fixative solution, Streck-STF, was purchased from Streck Labs, Omaha, NE. BrdU tissue staining kit from Zymed Labs, S. San Francisco, CA. All other reagents with the highest purity available, were purchased from Sigma Chemical Co.

2.2. Experimental Designs: Chemoprevention.

In a series of experiments, the chemopreventive effects of the selective COX-2 inhibitor, celecoxib, the nonselective COXs inhibitor, ibuprofen were studied. For comparison, the synthetic retinoid agent, 4-HPR was included.
2.2.1. Dietary and Tumor Induction protocols.

Chemoprevention with the nonselective NSAID, Ibuprofen: forty days old female Sprague Dawley rats were randomly assigned to three groups (20 rats/group) that were fed the control or the experimental diets. These diets were as follow: [1] the control diet: rat chow as powdered Teklad 22/5 rodent diet (W):8640; [2] the ibuprofen diet: powdered Teklad chow supplemented with 1000 mg ibuprofen/ kg diet (1000ppm); [3] the 4-HPR diet: powdered Teklad chow supplemented with 1.5 mmol 4-HPR/ kg diet. The 4-HPR was first dissolved into a vehicle consisting of ethanol:tricaprylin (1:4 v/v) plus 2% (w/v) of α-tocopherol (Abou-Issa, 1997), then thoroughly mixed with the powdered chow. The same amount of that vehicle was also added to the control and ibuprofen diets (25 ml/2 kg of the powdered chow) in order to permit direct comparisons among all dietary groups. The control and experimental diets were continued for the four months duration of the experiment.

Chemoprevention with the selective COX-2 blocking NSAID, Celecoxib: forty days old female Sprague Dawley rats were randomly assigned to one of three treatment groups (40 rats/group). The control group received powdered Teklad rodent diet, the celecoxib group received standard diet supplemented with 1500 mg celecoxib/ kg diet (1500 ppm), and the ibuprofen group received standard diet supplemented with 1500 mg ibuprofen/ kg diet of (1500 ppm). The control and experimental diets were continued for 105 days when the experiment was terminated.

COX-2 Blockade: Dose-Response Evaluation: forty days old female Sprague Dawley rats were randomly assigned to five experimental groups (20 rats/group) that were fed the control or the experimental diets. These diets were designed to evaluate the dose-response effect of
250, 500, 1000 and 1500 ppm of celecoxib given continuously throughout the experiment duration. These diets were as follow: [1] the control diet: powdered Teklad rodent diet; [2] Teklad chow supplemented with 250 ppm of celecoxib; [4] Teklad chow supplemented with 500 ppm of celecoxib; [4] Teklad chow supplemented with 1000 ppm of celecoxib; and [5] Teklad chow supplemented with 1500 ppm of celecoxib. The control and the experimental diets were continued for 122 days, when the experiment was terminated.

In the above-mentioned experiments, after the animals have received the diets for 7 days, each animal was intubated with a single intragastric dose of 15 mg of DMBA (7,12-dimethylbenz(a)anthracene) in 1.0 ml sesame oil using a feeding needle (Popper & Sons, New York, N.Y.).

**COX-2 Blockade: Anti-Initiation and Anti-Promotion Phases Evaluation:** forty days old female Sprague Dawley rats were randomly assigned to four groups (20 rats/group) that were fed the control or the experimental diets. At 50 days of age, all rats were given a single dose of 15 mg DMBA in 1.0 ml sesame oil by gavage using a feeding needle. For the evaluation of celecoxib chemopreventive effect on the initiation (I), promotion (P) phases as well as the combined initiation + promotion (I+P) phase, four regimes of diet (control and 3 experimental diets) were required.

These dietary regimes were fed to the animals as follows: [1] the control group was fed a control diet (powdered Teklad rodent chow) from 10 days before DMBA intubation until the end of the experiment; [2] the anti initiation phase effect of celecoxib was tested by feeding a Teklad chow supplemented with 1500 ppm of celecoxib from 10 days before to 10 after DMBA intubation. The rats were then fed the control Teklad chow without the
celecoxib supplement after this 20-day period onto the end of the experiment; [3] the anti promotion phase effect of celecoxib was tested by feeding the control chow without celecoxib from 10 days before till 10 days after DMBA intubation. Following this 20-day period, the animals were fed the Teklad chow supplemented with 1500 ppm of celecoxib till the end of the experiment; and [4] the anti initiation + promotion phase effects were tested by feeding the 1500 ppm celecoxib-supplemented diet from 10 days before DMBA intubation till the end of the experiment. These diets were continued until the experiment was terminated at 122 days post DMBA intubation.

All the additives were thoroughly blended into the powdered chow diets using a Hobart food mixer, and the foods were replaced with freshly prepared diets weekly. The diets were fed in stainless steel feeders designed with food hoppers. The rats were allowed food and water ad libitum. They were housed four/cage and maintained at 25 ° C, with 12 hours light/dark cycle in a climate-controlled room.

2.2.2. Outcome Measurements.

Both food consumption and rat weight gains were measured weekly throughout the duration of the experiments in addition to monitoring general health status and signs of toxicity due to treatments. Beginning at 28 days post DMBA intubation, the animals were palpated twice weekly to detect the presence and location of mammary tumors.

The time of appearance of each tumor (latency period), as well as the relative size and location of every tumor were recorded. Also the number of rats with tumors (incidence) and the number of tumors/ rat (tumor burden/multiplicity) were recorded in tabular form on a weekly basis and at the end of the study. This monitoring was continued until completion
of the experiment. At the termination of the experiment and just prior to sacrifice the animals, each tumor diameters were measured by a micrometer caliper, and the tumor volume was calculated using the formula $V = \frac{4}{3} \pi r^3$ where $r$ (radius) is half the average of the largest diameter and the diameter at right angle to it (Steel, 1977).

2.2.3. Specimens Collection.

The animals were sacrificed using CO$_2$ euthanasia. Necropsy included gross examination of all internal organs including the stomach, intestine, kidneys, and liver. At the time of sacrifice, blood samples were collected from each animal fed the control or experimental diets and sera were separated and rapidly frozen until assayed for agents' level using high performance liquid chromatography (HPLC).

All tumors were excised at the end of the experiment, and the tumor specimens were divided for histological evaluation (fixed in 10% neutral buffered formalin, processed, embedded in paraffin blocks, and processed for histological evaluation by routine procedures with H & E staining); for gene expression analyses (snap frozen and stored in liquid nitrogen [LN$_2$] until RT-PCR/blotting analyses were performed), and for immunohistochemical (IHC) analyses (Fixed in 10% neutral buffered formalin for 2 hours, washed in 0.2 M phosphate buffer, then stored at 4°C in 0.2 M phosphate buffer until processed for COX isozymes IHC staining) or (fixed overnight in 10% neutral buffered formalin at 4°C, then washed in 70% ethanol and stored at 4°C in 70% ethanol until COX isozymes IHC analyses). In addition to tumors samples, the stomach, intestine, and kidneys of each animal were resected and fixed in 10% neutral buffered formalin, processed and embedded in paraffin blocks, and processed for histological evaluation by routine procedures with H & E staining. Both
ovaries were also resected, snap frozen and stored in liquid nitrogen [LN₂] until gene expression analyses were performed.

2.3. Experimental Designs: Chemotherapy.

In a series of preclinical efficacy studies, the chemotherapeutic effects of the selective COX-2 inhibitor, celecoxib, the non selective general COXs inhibitor, ibuprofen, and the experimental selective COX-1 inhibitor, SC560 were evaluated.

2.3.1. Tumor Induction and Dietary protocols.

The mammary tumors were induced by intragastric intubation of 50 day old female Sprague Dawley rats with a single dose of 15 mg DMBA in 1.0 ml sesame oil/ rat using a feeding needle. The rats were maintained on powdered Teklad 22/5 rodent chow diet (W):8640, and allowed food and water *ad libitum*. Approximately four months later, the rats which have developed palpable tumors were randomly assigned to control and treatments groups, each with ten tumor-bearing rats. The rats were housed 3-4/ cage and maintained at 25°C, with 12 hours light/ dark cycle in a climate-controlled room.

*Chemotherapy with the nonselective NSAID, ibuprofen:* the control rats continued to receive the powdered chow diet, while the treatment group received the powdered chow supplemented with 1000 mg ibuprofen/ kg diet (1000 ppm), for the duration of 5 weeks.

*Chemotherapy with the selective COX-2 blocking NSAID, celecoxib:* the control rats continued to receive the powdered diet, while the treatment group received the powdered diet supplemented with 1500 mg celecoxib/ kg diet (1500 ppm), for the duration of 6 weeks.
Chemotherapy with Selective COX-1 or COX-2 blocking NSAIDs, SC560 or celecoxib: the control animals continued to receive the powdered diet, while the COX-2 inhibitor (SC560) or the COX-2 inhibitor (celecoxib) treated rats received the powdered diet supplemented with 130 mg SC-560/kg diet (130 ppm), or 1500 mg celecoxib/kg diet (1500 ppm) respectively, for the duration of 6 weeks.

All the additives were thoroughly blended into the powdered chow diets using a Hobart food mixer, and the foods were replaced with freshly prepared diets every week and administered in stainless steel feeders designed with food hoppers. The rats were allowed food and water ad libitum.

2.3.2. Outcome Measurements.

Food consumption was determined once weekly, and the average daily consumption/rat was estimated. Animals were weighed weekly, monitored for general health status and signs of toxicity due to treatment.

Initial baseline measurements of tumor size, tumor numbers, and rat body weight were determined just prior to commencement of treatment; final measurements were recorded just prior to termination of the experiment. The animals were palpated for tumors twice per week, and tumor perpendicular diameters were measured weekly by a micrometer caliper. Tumor volume was calculated using the formula \( V = \frac{4}{3} \pi r^3 \) where \( r \) is the average radius of these two perpendicular diameters.

2.3.3. Specimens Collection.

The animals were sacrificed using CO\(_2\) euthanasia. Necropsy included gross examination of all internal organs including the stomach, intestine, kidneys, and liver. At
the time of sacrifice, blood samples were collected from each animal and sera were separated and rapidly frozen until assayed for agents' level using high performance liquid chromatography (HPLC).

All tumors were excised at the end of the experiment, and the tumor specimens were divided for histology (fixed in 10% neutral buffered formalin, processed and embedded in paraffin blocks, and processed for histological evaluation by routine procedures with H&E staining); for gene expression analyses (snap frozen and stored in liquid nitrogen [LN₂] until RT-PCR/blotting analyses were performed); and for COX isoforms immunohistochemical (IHC) analyses (fixed in 10% neutral buffered formalin for 2 hours, washed in 0.2 M phosphate buffer, then stored at 4°C in 0.2 M phosphate buffer until processed for COXs IHC analyses). In addition to tumors samples, the stomach, intestine, and kidneys of each animal were resected and fixed in 10% neutral buffered formalin, processed and embedded in paraffin blocks, and processed for histological evaluation by routine procedures with H&E staining. For analysis of cellular proliferation (DNA synthesis), a protocol for labeling proliferating cells in tissue in vivo using the DNA synthesis marker, Bromo-deoxyUridine (BrdU) was performed in the COX-1 vs. COX-2 inhibition experiment. Each animal was injected intraperitoneally with 5 ml of (3mg/ ml) BrdU / PBS solution (3 mg BromodeoxyUridine/ 1 ml phosphate buffered saline). Three hours later the animals were euthanasied with CO₂ inhalation. In each rat, all tumors and control tissues (consist of a part of the jejunum) were excised and fixed in Streck STF for 24 hours, then processed into paraffin blocks. The pieces of a tumor and the control small bowel tissues were embedded in the same paraffin block prepared for immunostaining of BrdU labeled tissues. The
immunohistochemical staining was performed as per Zymed (Cat # 93-3944) BrdU Cell Proliferation Detection Kit.

2.4. Statistical Analyses.

The data bases were created using MS-Access and MS-Excel software programs. The data sets were analyzed with SAS, Minitab, and NCSS statistical software programs. The statistics on tumor latency, tumor incidence, tumor burden, tumor volume, and body weights were obtained using the univariate and means procedures. The statistical significance of comparisons between the experimental groups were obtained using Chi-square and proportion analysis tests, Fisher's exact test, t-test and paired t-test, analysis of variance (ANOVA) and multiple mean comparison procedures, ANOVA with repeated measures procedure, survival analysis procedures, and linear regression and correlation procedures. When the assumptions of the model were not met, non parametric statistical procedures were used. Sample size used in these experiments were determined by power calculations (α = 0.05, β = 0.20) to detect at least 30% difference between the experimental groups. The measurements are reported as proportion (percentage), and mean ± SEM (standard error of the mean). Statistical significance was accepted when p<0.05 (Woolson, 1987; Kahn et al., 1989; Glantz et al., 1990; Elston et al., 1994; Hollander et al., 1994; Collett, 1994).
CHAPTER 3

RESULTS


3.1.1 Chemoprevention with the nonselective NSAID, ibuprofen:

*Mammary Tumors Data:* The results of this experiment testing the chemopreventive effects of ibuprofen and 4-HPR on the time course of mammary tumor development in DMBA-treated rats are shown in Figures 3.1 and 3.2, and summarized in Table 3.1. The administration of ibuprofen (1000 mg/kg diet) or 4-HPR (1.5 mmol/kg diet) has prolonged the latency period of mammary tumor induction. In rats that received the control diet only, the mean duration until the appearance of the first tumor was 67 days post DMBA intubation. On the other hand, the tumor induction period was markedly delayed in the ibuprofen and retinoid treated rats. The mean latency periods observed were 77 and 87 days in the ibuprofen and 4-HPR treated rats, respectively. Thus 4-HPR caused almost 30% increase in tumor latency period as compared to the control group (p<0.05), while ibuprofen showed a 15% delay compared to the control.

Both ibuprofen and 4-HPR showed a chemopreventive activity relative to the control group in terms of both the percentage of rats with tumors (tumor incidence) and the average
number of tumors/rat (tumor burden/multiplicity). In the control group, 89% of the rats had mammary tumors by 112 days post DMBA intubation. However, tumor incidence at the same time was 74% for the ibuprofen treated rats which represents an almost 17% inhibition of tumor incidence compared to controls. The retinoid 4-HPR was more potent and showed a tumor incidence of 62% at 112 days post DMBA intubation. This value represents approximately 30% inhibition in tumor incidence as compared to control group (p < 0.04).

Similarly, when tumor burden (multiplicity), expressed as the average number of tumors/rat, was measured, ibuprofen and 4-HPR caused an almost 37% and 35% reduction in mammary tumor burden as compared to the control rats, respectively. At 112 days post DMBA intubation, the control rats had an average of 2.26 tumors/rat compared to 1.42 and 1.46 tumors/rat in animals receiving the ibuprofen and 4-HPR diets, respectively. The difference among the three groups was statistically significant (p < 0.04).

Tumor sizes (in cm^3) were measured in tumor-bearing rats at the end of the experiment just before the sacrifice of the rats. Tumor volumes were also significantly decreased to the same extent in the ibuprofen and 4-HPR treated rats, as compared to the control group. At 112 days post DMBA intubation, the average tumor volumes in cm^3 were 3.25, 0.86, and 0.83 for the control, ibuprofen and 4-HPR treated rats, respectively. Thus animals treated with ibuprofen or 4-HPR showed almost 74% and 75% lower mean tumor volumes than the control group. The difference among the three groups was again statistically significant (p < 0.04).

**Histopathologic Data:** The pathological evaluation of all mammary tumors excised at the completion of the experiment showed histological features consistent with adenocarcinoma
Figure 3.1. Treatment effects on the time-course change in mammary tumor incidence. The Control group was fed Teklad chow diet; Ibuprofen group was fed powdered chow + 1000 mg Ibuprofen / kg diet; and 4HPR group was fed powdered chow + 1.5 mmol 4-HPR / kg diet.
Figure 3.2: Treatment effects on the time-course change in mammary tumor burden.
The Control group was fed Teklad chow diet; Ibuprofen group was fed powdered chow + 1000 mg Ibuprofen / kg diet; and 4HPR group was fed powdered chow + 1.5 mmol 4-HPR / kg diet.
<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Mean Latency (days)</th>
<th>Tumor Incidence (% rats with tumors)</th>
<th>Tumor Burden (mean no. of tumors/ rat ± SE)</th>
<th>Tumor Volume (cm³) (mean volume ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>67</td>
<td>89</td>
<td>2.26 ± 0.48</td>
<td>3.25 ± 1.86</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>77</td>
<td>74 (17)*</td>
<td>1.42 ± 0.30 (37)*</td>
<td>0.86 ± 0.22 (74)*</td>
</tr>
<tr>
<td>4-HPR</td>
<td>87</td>
<td>62 (30)*</td>
<td>1.46 ± 0.49 (35)*</td>
<td>0.83 ± 0.21 (75)*</td>
</tr>
</tbody>
</table>

Table 3.1: Effects of Ibuprofen and 4-HPR on the development and growth of DMBA-induced rat mammary tumors.

- Twenty rats/group were fed powdered Teklad rodent chow diet, supplemented with 1000 mg and 1.5 mmol/kg diet of Ibuprofen and 4-HPR, respectively. The data represent the rats at 16 weeks after DMBA intubation.

- Percent inhibitions in incidence rates, tumor burden, and tumor volume for the experimental diets relative to the control diet are given in parenthesis. % Inhibition = \( \frac{\text{Value of control rats} - \text{Value of treated rats}}{\text{Value of control rats}} \times 100 \)

- Difference between the groups \( p < 0.04 \)

- Relative to the control group \( p < 0.04 \)
of the mammary gland. There was marked heterogeneity in tumors isolated from control rats intubated with DMBA and provided with standard control diet. Although all the tumors were adenocarcinomas, they contained a heterogeneous morphological patterns consisting of five predominant histological types including adenoid cystic carcinoma, solid medullary carcinoma, papillary carcinoma, follicular and pseudo follicular carcinoma and myxoid carcinoma. There was, however, a corresponding decrease in tumor heterogeneity and tumor grade in the tumors excised from rats treated with 4-HPR or ibuprofen. Regarding COX isozymes gene expression, molecular studies performed on samples from this investigation have shown that the gene expression of both COX-1 and -2 were inhibited by ibuprofen and 4-HPR. In addition, localization studies of COXs immunoreactive proteins have indicated more localization of COX-1 immunospecific protein to the inflammatory cells in the stroma, and more localization of COX-2 immunospecific protein within tumor cells.

**General Observations:** Weight loss caused by reduced food intake due to treatments’ toxicity may affect tumor growth (Klurfeld et al., 1989). Therefore animals in the three groups were weighed weekly, and their initial and final weights are shown in Table3.2. The prolonged feeding of diets supplemented with ibuprofen (1000 mg / kg diet) or 4-HPR (1.5 mmol/ kg diet) did not significantly affect the weight gain of the rats throughout the entire duration of the experiment (p< 0.19). Food intake was almost identical in all three groups. No sign of toxicity or adverse health effects were observed in the ibuprofen or retinoid treated animals. Administration of ibuprofen or 4-HPR did not produce gross changes in the liver, kidneys, stomach, or intestinal tract.
<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Body Weight (gm) *</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial *</td>
<td>Final *</td>
<td></td>
</tr>
<tr>
<td>Control (Chow+Vehicle)</td>
<td>127.90 ± 0.94</td>
<td>254.28 ± 3.02</td>
<td></td>
</tr>
<tr>
<td>Ibuprofen (1000 mg/kg diet)</td>
<td>126.84 ± 0.74</td>
<td>249.94 ± 4.19</td>
<td></td>
</tr>
<tr>
<td>4-HPR (1.5 mmol/kg diet)</td>
<td>127.34 ± 0.80</td>
<td>243.76 ± 4.22</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2: Effect of Ibuprofen and 4-HPR diets on body weights.

* Value = Mean ± SEM

* Difference between treatment groups weights: p < 0.19
3.1.2 Chemoprevention with the selective COX-2 blocking NSAID, celecoxib:

**Mammary Tumor Data:** The chemopreventive effects of celecoxib and ibuprofen on mammary tumor development are shown in Figures 3.3 and 3.4, and summarized in Table 3.3. In the control group 100% of animals have developed malignant tumors, and the majority of animals (95%) had multiple tumors, and the mean tumor size was much greater (1.5 cm³). In contrast, the specific COX-2 inhibitor, celecoxib (1500 ppm), has produced significant reductions (p < 0.001) in the incidence of mammary cancer (67%), tumor burden (87%), and tumor volume (83%) compared to the control group. In the celecoxib treated group, only 13 of 40 animals (33%) developed malignant tumors, and three animals have developed fibroadenomas; and the mean tumor volume was relatively small (0.26 cm³).

The weaker COX-2 blocker, ibuprofen (1500 ppm), also produced statistically significant reductions in cancer risk, tumor burden and size (40%, 52%, and 57%, respectively) relative to the control group (p < 0.001), but its effects were of lesser magnitude than celecoxib. The administration of NSAIDs also prolonged the latency period of tumor induction. In animals receiving the control diet only, mean detection occurred at 58 days post DMBA induction, compared to 95 and 86 days in the celecoxib and ibuprofen treatment groups, respectively.

These results reflect strong suppression of mammary carcinogenesis (67% inhibition of breast cancer incidence) by the specific COX-2 inhibitor, celecoxib, and intermediate suppression (40% inhibition of incidence) by the general NSAID, ibuprofen.

**Histopathologic/Pharmacologic Data:** At the completion of the experiment, 127 palpable mammary tumors were excised from control rats, 61 from rats receiving ibuprofen, and 18
Figure 3.3: Treatment effects on the time-course change in mammary tumor incidence.

The Control group was fed Teklad chow diet; Celecoxib group was fed powdered chow + 1500 mg Celecoxib/ kg diet; Ibuprofen group was fed powdered chow + 1500 mg Ibuprofen / kg diet.
Figure 3.4: Treatment effects on the time-course change in mammary tumor burden. The Control group was fed Teklad chow diet; Celecoxib group was fed powdered chow + 1500 mg Celecoxib/kg diet; Ibuprofen group was fed powdered chow + 1500 mg Ibuprofen/kg diet.
<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Mean Latency (Days)</th>
<th>Tumor Incidence (% rats with tumor)</th>
<th>Tumor Burden (Mean No. of Tumors/ Rat + SE)</th>
<th>Tumor Volume (cm$^3$) (mean volume ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>58</td>
<td>100</td>
<td>3.18 ± 0.22</td>
<td>1.50 ± 0.45</td>
</tr>
<tr>
<td>Celecoxib: All Tumors</td>
<td>95 $^b$</td>
<td>40 (60)$^*$$^b$</td>
<td>0.45 ± 0.09 (86)$^*$  $^b$</td>
<td>0.29 ± 0.11 (81)$^*$$^b$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33 (67)$^*$$^b$</td>
<td>0.41 ± 0.10 (87)$^*$$^b$</td>
<td>0.26 ± 0.11 (83)$^*$$^b$</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>86 $^b$</td>
<td>60 (40)$^*$$^b$</td>
<td>1.53 ± 0.28 (52)$^*$$^b$</td>
<td>0.64 ± 0.19 (57)$^*$$^b$</td>
</tr>
</tbody>
</table>

Table 3.3: Effects of Celecoxib and Ibuprofen on the development and growth of DMBA-induced rat mammary tumors.

* Forty rats randomly assigned to each experimental group, were fed powdered Teklad rodent diet supplemented with 1500 mg/ kg diet (1500 ppm) of Celecoxib or Ibuprofen. The data represent the rats at 15 weeks post DMBA intubation,

* Percent Inhibitions in incidence rates, tumor burden, and tumor volume for the experimental diets relative to the control diet are given in parentheses.

$^b$ Relative to the control group: $p < 0.001$
from rats receiving celecoxib. The histopathologic evaluation have revealed that all tumors from the control and ibuprofen groups were adenocarcinomas. Of the eighteen tumors excised from animals receiving celecoxib, fifteen were adenocarcinomas and three were nonmalignant fibroadenomas. The serum levels of celecoxib assayed by HPLC ranged from 1.5 to 2.2 μg/ml, with a mean of 1.75 μg/ml. The mean level was significantly higher in rats without tumors versus those with tumors (2.1 vs. 1.63 μg/ml, respectively, p<0.01). Serum levels of ibuprofen ranged from 6 to 10 μg/ml, with a mean of 8 μg/ml.

**General Observations:** The animals in the three groups were weighed weekly, and their initial and final weights are shown in Table 3.4. Average body weights of animals in the three treatment groups were similar throughout the experiment. Administration of celecoxib or ibuprofen did not produce any gross or histological changes in the liver, kidneys, stomach, or intestinal tract.
<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Body Weight (gm) *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial *</td>
</tr>
<tr>
<td>Control</td>
<td>144.10 ± 1.22</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>147.56 ± 1.26</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>148.11 ± 2.59</td>
</tr>
</tbody>
</table>

Table 3.4: Effects of Celecoxib and Ibuprofen diets on body weights.

* Value = Mean ± SEM

* Difference among the experimental groups : P < 0.24
** Difference among the experimental groups : P < 0.16
3.1.3. COX-2 Blockade: Dose-Response Evaluation:

**Mammary Tumor Data:** The chemopreventive effects of the various doses of celecoxib (250, 500, 1000, and 1500 ppm) on mammary tumor development and growth are shown in Figures 3.5 and 3.6, and are summarized in Tables 3.5 and 3.6. The administration of celecoxib prolonged the latency periods of mammary cancer induction. In rats that received the control diet, the mean duration until the first tumor appearance was 71 days post DMBA intubation. In contrast, the mean latency periods were 94, 95, and 105 days in the rats receiving 500, 1000, 1500 ppm of celecoxib respectively (p<0.05). The 250 ppm dose of celecoxib caused a delay in the mean latency period but of lesser magnitude (78 days versus 71 days in the control rats).

The administration of increasing doses of celecoxib also significantly inhibited mammary tumor incidence. At 122 days post DMBA intubation, mammary tumor incidence was 100% in the control rats compared to 80%, 50%, 45% and 25% in rats receiving 250, 500, 1000 and 1500 ppm celecoxib, respectively (p<0.001). Tumor incidence inhibition ranged from 20% to 75% when compared with the incidence in the control rats. When analyzed for celecoxib’s dose-effect, these results have yielded a statistically significant inverse correlation coefficient of \( r = -0.93 \) (p<0.02), indicating a significant dose-dependent inhibition of tumor incidence with increasing levels of celecoxib in the diet.

Similarly, when tumor burden (multiplicity), expressed as the average number of tumors/rat was measured, celecoxib treatment also caused a dose-dependent inhibition of tumor burden (multiplicity) as compared to the control rats. At 122 days post DMBA intubation, the control rats had an average of 3.46 tumors/rat compared to 1.80, 1.00, 0.75
Figure 3.6: Treatment effects on the time-course change in mammary tumor burden. The Control group was fed Teklad chow diet; Celecoxib groups were fed powdered chow + 1500 mg, 1000 mg, 500 mg, and 250 mg Celecoxib/ kg diet, respectively.
Figure 3.5: Treatment effects on the time-course change in mammary tumor incidence.
The Control group was fed Teklad chow diet; Celecoxib groups were fed powdered chow + 1500 mg, 1000 mg, 500 mg, and 250 mg Celecoxib/kg diet, respectively.
<table>
<thead>
<tr>
<th>Treatment Groups *</th>
<th>Mean Latency (Days) b</th>
<th>Tumor Incidence (% rats with Tumors) c</th>
<th>Tumor Burden (Mean No. of tumors/ rat ± ES) c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>71</td>
<td>100</td>
<td>3.46 ± 0.40</td>
</tr>
<tr>
<td>Celecoxib 250</td>
<td>78</td>
<td>80 (20)*</td>
<td>1.80 ± 0.32 (48)*</td>
</tr>
<tr>
<td>Celecoxib 500</td>
<td>94</td>
<td>50 (50)*</td>
<td>1.00 ± 0.29 (71)*</td>
</tr>
<tr>
<td>Celecoxib 1000</td>
<td>95</td>
<td>45 (55)*</td>
<td>0.75 ± 0.22 (78)*</td>
</tr>
<tr>
<td>Celecoxib 1500</td>
<td>105</td>
<td>25 (75)*</td>
<td>0.50 ± 0.19 (86)*</td>
</tr>
</tbody>
</table>

Table 3.5: Effects of Celecoxib diets on the Incidence and Tumor Burden of DMBA-induced mammary tumors.

* Twenty rats/group were fed powdered Teklad rodent diet, supplemented in the experimental groups with 250, 500, 1000, and 1500 mg Celecoxib/kg diet, respectively. The data represent the rats at 17 weeks post DMBA-intubation.

* Percent inhibitions in incidence rates, and tumor burden for the experimental diets relative to the control diet are given in parenthesis.

b Linear trend : p < 0.05
c Linear trend : p < 0.001
<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Tumor Volume (cm$^3$) (Mean volume ± SE) *</th>
<th>Celecoxib Steady-State Serum Levels (µg/ml) (Mean Value ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.29 ± 0.36</td>
<td>ND b</td>
</tr>
<tr>
<td>Celecoxib 250</td>
<td>0.42 ± 0.10 (67) *</td>
<td>0.22 ± 0.07</td>
</tr>
<tr>
<td>Celecoxib 500</td>
<td>0.34 ± 0.18 (74) *</td>
<td>0.54 ± 0.05</td>
</tr>
<tr>
<td>Celecoxib 1000</td>
<td>0.31 ± 0.14 (76) *</td>
<td>0.85 ± 0.13</td>
</tr>
<tr>
<td>Celecoxib 1500</td>
<td>0.16 ± 0.07 (88) *</td>
<td>1.70 ± 0.34</td>
</tr>
</tbody>
</table>

Table 3.6: Effects of Celecoxib diets on Volume of DMBA-induced mammary tumors

* Percent inhibitions in tumor volume for the experimental diets relative to the control diet are given in parenthesis.

a Linear trend : $p < 0.03$

b ND : Not detected.
and 0.50 tumors/rat in the animals receiving 250, 500, 1000 and 1500 ppm celecoxib diets, respectively. The difference between the groups was statistically significant (p<0.001).

Tumor volumes were measured in tumor bearing rats at the end of the experiment and just immediately before the sacrifice of the animals. Tumor volumes were also significantly reduced, in a dose-dependent manner, by increasing the dose of celecoxib from 250 to 1500 ppm in the diet. At 122 days post DMBA intubation average tumor volumes in the rats fed 250, 500, 1000 and 1500 ppm celecoxib were 0.42, 0.34, 0.31, 0.16 cm³, respectively, compared to 1.29 cm³ in the control rats (Table 3.6). The differences between the experimental groups were also statistically significant (p<0.03). Inhibition of tumor volumes, when analyzed for celecoxib’s dose-effect, have yielded an inverse correlation coefficient of \( r = -0.96 \) (p<0.005), indicating a significant dose-dependent decrease in tumor volumes with increasing levels of celecoxib in the diet.

**Histopathologic/Pharmacologic Data:** The histopathological evaluation have revealed that all tumors from the control rats and animals fed the 250, 500, 1000 ppm celecoxib were adenocarcinomas. Of the 6 tumors excised from animals receiving 1500 ppm celecoxib, 5 were adenocarcinomas and 1 was a nonmalignant fibroadenoma. There was marked heterogeneity in tumors isolated from rats intubated with DMBA and provided with control diet, while tumor heterogeneity and tumor grade was markedly decreased in the celecoxib treated animals. Regarding COX-2 gene expression, the molecular studies performed on samples from this investigation have shown that COX-2 to be expressed in both the malignant mammary epithelial cells, and is also focally detected in the inflammatory cells and vasculature within and immediately adjacent to the tumorous regions.
Steady-state serum levels of celecoxib in animals receiving increasing doses of the agent and assayed by HPLC (Table 3.6) were as follows: [a] mean of 250 ppm celecoxib is 0.22 (± 0.07 as SEM) μg/ml; [b] mean of 500 ppm celecoxib is 0.54 (± 0.05 as SEM) μg/ml; [c] mean of 1000 ppm celecoxib is 0.85 (± 0.13 as SEM) μg/ml; and [d] mean of 1500 ppm celecoxib is 1.70 (± 0.34 as SEM) μg/ml. Celecoxib levels were not detectable in sera of control rats.

General Observations: The animals fed the control or experimental diets were weighed weekly, and their initial and final weights are shown in Table 3.7. Prolonged feeding of diets supplemented with celecoxib did not significantly affect the weight gain of the rats throughout the entire duration of the experiment (p<0.57). Food intake was almost identical in all groups. No signs of toxicity, nor gross or histological changes in the liver, kidneys, stomach or intestine were observed, even in the animals treated with 1500 ppm celecoxib.
<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Body Weight (gm) *</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial *</td>
<td>Final **</td>
</tr>
<tr>
<td>Control</td>
<td>144.53 ± 1.09</td>
<td>255.19 ± 3.28</td>
</tr>
<tr>
<td>Celecoxib 250 mg</td>
<td>142.87 ± 0.93</td>
<td>250.92 ± 2.43</td>
</tr>
<tr>
<td>Celecoxib 500 mg</td>
<td>144.63 ± 1.19</td>
<td>259.26 ± 4.06</td>
</tr>
<tr>
<td>Celecoxib 1000 mg</td>
<td>143.97 ± 1.23</td>
<td>256.42 ± 3.76</td>
</tr>
<tr>
<td>Celecoxib 1500 mg</td>
<td>145.37 ± 0.95</td>
<td>257.92 ± 2.27</td>
</tr>
</tbody>
</table>

Table 3.7: Effects of Celecoxib diets on body weights.

* Value = Mean ± SEM

* Difference between the experimental groups: p < 0.57

** Difference between the experimental groups: p < 0.47
3.1.4. COX-2 Blockade: Anti-Initiation and Anti-Promotion Phases Evaluation:

*Mammary Tumors Data:* The results of this experiment testing the chemopreventive effects of COX-2 inhibitor, celecoxib, on the initiation (I) phase, promotion (P) phase, and the initiation + promotion (I+P) phase of DMBA-induced mammary carcinogenesis are shown in Figures 3.7 and 3.8, and are summarized in table 3.8. The dietary regimen for the control group consisted of Teklad chow diet only, while the three celecoxib groups were fed the chow diets supplemented with 1500 ppm of celecoxib. The administration of celecoxib significantly prolonged the mean latency period of mammary tumor development as compared to the control. In animals receiving the control diet only, the mean induction period was 71 days post DMBA intubation compared with 96, 85 and 105 days in the celecoxib treated animals tested during the initiation, promotion and initiation+ promotion phases of mammary cancer, respectively. There were statistically significant differences in the mean tumor latency of the celecoxib treated groups as compared to the control group (p<0.001). The initiation+promotion together, significantly showed the longest mean latency period, as compared to either the initiation phase or the promotion phase alone.

At 122 days post DMBA intubation, tumor incidence was 100% in the control group. In contrast, in the celecoxib-feeding groups tumor incidences were markedly reduced to 55%, 60% and 25% for the initiation, promotion and initiation+promotion phases, respectively (p<0.001). Celecoxib has inhibited tumor incidence in the initiation and promotion phases approximately to the same extent, and the combined effect (initiation+promotion) was approximately additive in the initiation+promotion phase group.

Similarly, when the average number of tumors/rat (tumor burden/multiplicity) was
assessed, celecoxib has caused marked reduction in tumor burden (multiplicity) as compared to the control group. At 122 days post DMBA intubation, the average number of tumors/rat (tumor burden/multiplicity) was 3.4 in the control group, whereas the average number of tumors/rat was decreased to 0.8, 1.0 and 0.5 tumors/rat in animals receiving celecoxib during the initiation, promotion or initiation+promotion phases, respectively (p<0.001).

Tumor volumes measured immediately before the sacrifice of the rats were 0.53, 0.55 and 0.16 cm³ for the rats fed celecoxib during initiation, promotion or initiation + promotion phases respectively, compared to 1.29 cm³ for the control rats (p<0.003).

These results show that celecoxib acts on the initiation or promotion phases almost to the same extent, with the effect being maximal when fed during the combined initiation + promotion phase of mammary carcinogenesis.
Figure 3.7: Treatment effects on the time-course change in mammary tumor incidence. The Control group was fed Teklad chow diet; Celecoxib groups were fed powdered chow + 1500 mg Celecoxib/kg diet.
Figure 3.8: Treatment effects on the time-course change in mammary tumor burden.
The Control group was fed Teklad chow diet; Celecoxib groups were fed powdered chow + 1500 mg Celecoxib/ kg diet.
<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Mean Latency (Days)</th>
<th>Tumor Incidence (% Rats with Tumors)</th>
<th>Tumor Burden (Mean No. of Tumors/Rat ± SE)</th>
<th>Tumor Volume (cm³) (Mean Volume ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (C)</td>
<td>71</td>
<td>100</td>
<td>3.46 ± 0.40</td>
<td>1.29 ± 0.36</td>
</tr>
<tr>
<td>Initiation Phase (I)</td>
<td>96</td>
<td>55 (45)*</td>
<td>0.80 ± 0.23 (77)*</td>
<td>0.53 ± 0.18 (59)*</td>
</tr>
<tr>
<td>Promotion (P)</td>
<td>85</td>
<td>60 (40)*</td>
<td>1.00 ± 0.23 (71)*</td>
<td>0.55 ± 0.20 (57)*</td>
</tr>
<tr>
<td>Initiation+Promotion (I+P)</td>
<td>105</td>
<td>25 (75)*</td>
<td>0.50 ± 0.19 (86)*</td>
<td>0.16 ± 0.07 (88)*</td>
</tr>
</tbody>
</table>

Table 3.8: Effects of Celecoxib on Initiation and Promotion Phases of DMBA-induced rat mammary tumors.

* Twenty rats/group were fed powdered Teklad rodent diet, supplemented in Celecoxib groups with 1500 mg of celecoxib/kg diet. The (I+P) group received celecoxib diet throughout the whole duration of the experiment. The (I) group received celecoxib diet for 10 days before and 10 days after DMBA intubation, while the (P) group received celecoxib diet 10 days post DMBA intubation and continued till the termination of the experiment. The data represent the rats at 17 weeks post DMBA-intubation.

* Percent inhibitions in incidence rates, tumor burden, and tumor volume for the experimental diets relative to the control diet are given in parenthesis.

\[ b \] Difference between the experimental groups: \( p < 0.001 \)

\[ c \] Difference between the experimental groups: \( p < 0.003 \)
**Histopathologic/Pharmacologic Data:** The histopathological evaluation has revealed that all tumors from the control rats and animals in the I, P phases or I+P phase were adenocarcinomas, except one tumor in the I+P phase which revealed histologic features consistent with fibroadenoma. There was marked heterogeneity in tumors excised from the control animals, whereas tumor heterogeneity and tumor grade were markedly decreased in the celecoxib treated animals.

The steady-state serum levels of celecoxib, collected at the end of the experiment and assayed by HPLC, were as follows: [a] I+P phase (1500 ppm celecoxib) the mean is 1.70 (± 0.34 as SEM) μg/ml; [b] P phase (1500 ppm celecoxib) the mean is 0.97 (± 0.44 as SEM) μg/ml. Celecoxib levels were not detectable in sera of control or the anti-initiation phase (I) animals.

**General Observations:** Prolonged feeding of diets supplemented with 1500 ppm celecoxib during the promotion or the initiation + promotion phases did not produce any gross or histological changes in the liver, kidneys, stomach or intestinal tract. Celecoxib treatment did not significantly affect (p>0.12) the weight gain of the rats throughout the duration of the experiment (Table 3.9). Food intake was almost identical in all the groups.
<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Body weight (gm) *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial *</td>
</tr>
<tr>
<td>Control (C)</td>
<td>144.53 ± 1.09</td>
</tr>
<tr>
<td>Initiation Phase (I)</td>
<td>143.11 ± 0.73</td>
</tr>
<tr>
<td>Promotion/Progression Phase (P)</td>
<td>146.47 ± 0.94</td>
</tr>
<tr>
<td>Initiation + Promotion/progression (I+P)</td>
<td>145.37 ± 0.95</td>
</tr>
</tbody>
</table>

Table 3.9: Effects of Celecoxib diet, initiated in different phases, on body weights.

* Value = Mean ± SEM

* Difference between the experimental groups : p < 0.12

** Difference between the experimental groups : p < 0.59
3.2. Chemotherapeutic Studies:

3.2.1. Chemotherapy with the nonselective NSAID, ibuprofen:

*Mammary Tumors data:* The results of this experiment testing the chemotherapeutic effects of ibuprofen on tumor volume and tumor numbers *vis-a-vis* cancer load (CL) of DMBA-induced mammary tumors are shown in Figure 3.9, and are summarized in Table 3.10. Rats with measurable mammary tumors were fed control diet or ibuprofen supplemented diet at a concentration of 1000 mg ibuprofen / kg diet (1000ppm), and the effects on tumor volume and number were measured and compared relative to the baseline and to the control rats.

Figure 3.9 depicts the trend in mean tumor volume over time in each treatment group as a function of feeding control or ibuprofen supplemented diets. There was a significant divergent trend in tumor volumes between the two groups (p<0.001), as mammary tumors continued to grow actively in the rats fed the control diet, whereas in the ibuprofen treated animals growth arrest was observed, and the average size of tumors was mildly regressed (Table 10). In the control animals, over four weeks of treatment, the average size of tumors increased by more than 200% relative to baseline; in contrast to an 18% reduction in the average tumor size relative to the baseline in the ibuprofen treated group (p<0.05). Tumor regression occurred in 70% of the rats receiving ibuprofen treatment. During the treatment period, six new mammary tumors appeared in the control and the ibuprofen groups, a 16 % increase relative to baseline.
Figure 3.9: Treatment effects on the time-course change in mammary tumor volume. The Control group was fed Teklad chow diet; Ibuprofen group was fed powdered chow + 1000 mg Ibuprofen/kg diet.
<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Mean Tumor Volume (cm$^3$)</th>
<th>Total Number of Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>Control</td>
<td>1.20 ± 0.03</td>
<td>2.76 ± 0.06</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>1.04 ± 0.04</td>
<td>0.85 ± 0.05</td>
</tr>
</tbody>
</table>

Table 3.10: Effects of Ibuprofen on growth of DMBA-induced rat mammary tumors.

* Ten rats with established tumors/ group were fed powdered Teklad rodent diet, supplemented in the Ibuprofen group with 1000 mg of ibuprofen/ kg diet. The data represent 4 weeks of ibuprofen treatment.

$^b$ Value = Mean ± SEM.

$^c$ The divergent trend over time in mean tumor volume between the experimental groups: p < 0.001

$^d$ Change from baseline: p < 0.05

$^*$ The number include 6 new tumors and one completely regressed tumor during the treatment period.
Histopathologic Data: Pathological evaluation of all tumors excised at the end of the study revealed histological features consistent with papillary adenocarcinoma of the mammary gland. Also the average tumors weight in the control group (6.7 gm) was significantly higher than the mean tumors weight (3.9 gm) in the ibuprofen treated group (p<0.01). Regarding COX isozymes gene expression, molecular studies performed on samples from this investigation have shown that gene expression of both COX-1 and -2 were inhibited by ibuprofen. Also localization studies of COXs immunoreactive proteins have indicated more localization of COX-1 immunospecific protein to the inflammatory cells in the stroma, and more localization of COX-2 immunospecific protein within tumor cells.

General Observations: Since weight gain and food consumption may affect tumor growth (Klurfeld, 1989), these variables as well as general health status were monitored throughout the experiment. The ibuprofen supplemented diet did not significantly affect the body weights of the rats (Table 3.11). On average, there were no significant differences in weight between the control and ibuprofen treated groups (p<0.30). Also there were no significant difference in food consumption between the two groups. Animals receiving ibuprofen displayed no clinical signs of toxicity, and the gross or histological examination of the liver, kidneys, stomach and intestine did not show any changes that would indicate toxicity.
<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Body Weight (gm)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>Control</td>
<td>250.49 + 4.95</td>
<td>263.18 + 5.24</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>250.25 + 3.19</td>
<td>258.87 + 7.88</td>
</tr>
</tbody>
</table>

Table 3.11: Effect of Ibuprofen diet on body weights.

- Value = Mean + SEM
- The data represent 5 weeks of Ibuprofen treatment.
- Difference between the two experimental groups weights: $p < 0.30$
3.2.2. Chemotherapy with selective COX-2 blocking NSAID, celecoxib:

*Mammary tumors' Data:* The rats with established DMBA-induced mammary tumors were fed either the standard control diet, or the COX-2 inhibitor, celecoxib supplemented diet for 6 weeks, at a concentration of 1500 mg celecoxib/kg diet, and the agent effects on tumor volume and number (cancer load) were measured and compared relative to the baseline and to the control rats.

Figure 3.10 shows the change in tumor volume in each group as a function of feeding control or celecoxib supplemented diets over the 6-week treatment period. Mammary tumors continued to grow actively in the rats fed the control diet, whereas in the animals given celecoxib the average size of the tumors regressed. There was a significant divergent trend in tumor volumes between the two experimental groups (p<0.001). In the control animals, the average size of tumors increased by more than 500% relative to baseline (p<0.001), in contrast to 32% reduction in the average tumor size in the celecoxib group (p<0.04) relative to baseline (Table 3.12). Tumor regression occurred in 90% of rats receiving celecoxib. During the treatment period, ten new mammary tumors appeared in the control group, a 30% increase (Table 3.12). In contrast, celecoxib supplemented diet significantly reduced the number of palpable mammary tumors from 24 to 18, a decrease of 25% over the same time period (p<0.05). There was no spontaneous regression of tumors in the control animals.
Figure 3.10: Treatment effects on the time-course change in mammary tumor volume. The Control group was fed Teklad chow diet; Celecoxib group was fed powdered chow + 1500 mg Celecoxib/kg diet.
Table 3.12: Effects of Celecoxib on growth of DMBA-induced rat mammary tumors.

<table>
<thead>
<tr>
<th>Experimental Groups *</th>
<th>Mean Tumor Volume (cm^3) ( b )</th>
<th>Total Number of Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>Control</td>
<td>0.28 ± 0.07</td>
<td>1.45 ± 0.43</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>0.19 ± 0.05</td>
<td>0.13 ± 0.05</td>
</tr>
</tbody>
</table>

* Ten rat with established tumors/ group were fed powdered Teklad rodent diet, supplemented in the Celecoxib group with 1500 mg of celecoxib/ kg diet. The data represent 6 weeks of celecoxib treatment.

\( b \) Value = Mean ± SEM.

\( c \) The divergent trend over time in mean tumor volume between the experimental groups: \( p < 0.001 \)

\( d \) The divergent trend over time in tumor numbers between the experimental groups: \( p < 0.01 \)

\( e \) Change from baseline: \( p < 0.05 \)
**Histopathologic/Pharmacologic Data:** The pathological evaluation of all mammary tumors excised at the completion of the experiment showed histological features consistent with adenocarcinoma of the mammary gland. The average celecoxib’s serum level in treated rats at the end of the experiment was $3.55 \mu g / ml \pm 0.41 \mu g$ as the SEM.

**General observations:** Weight gain and food consumption were monitored throughout the experiment. The celecoxib (1500ppm) supplemented diet did not significantly affect the body weights of the rats. On average, the control and celecoxib groups gained approximately the same weight (Table 3.13). Food intake was almost identical in the two experimental groups. Celecoxib treatment did not produce clinical, gross or histological changes in the examined organs of the rats that would indicate toxicity.
<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Body Weight (gm)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial*</td>
<td>Final **</td>
</tr>
<tr>
<td>Control (Chow + Vehicle)</td>
<td>259.1 ± 4.04</td>
<td>269.7 ± 5.28</td>
</tr>
<tr>
<td>Celecoxib (1500 mg/kg diet)</td>
<td>255.8 ± 3.88</td>
<td>265.4 ± 4.37</td>
</tr>
</tbody>
</table>

Table 3.13: Effect of Celecoxib diet on body weights.

* Value = Mean ± SEM

* Difference between the two groups weights : p < 0.50

** Difference between the two groups weights : p < 0.50
3.2.3. Chemotherapy with selective COX-1 or COX-2 blocking NSAIDs, SC560 or celecoxib:

Mammary Tumors data: Two selective NSAIDs, COX-2 inhibitor, celecoxib and COX-1 inhibitor, SC-560 were tested at a concentration of 1500 ppm and 130 ppm, respectively. The agents effects on tumor volume and number (cancer load) were measured and compared relative to the baseline and to the control rats.

Figure 3.11 shows the changes in tumor volume over time for each experimental group. There was significant divergent trend in mean tumor volume overtime for the experimental groups (p<0.01) as mammary tumors continued to grow actively in the rats fed the control or the SC-560 diets, whereas in animals receiving celecoxib supplemented diet the average size of tumors mildly regressed (Table 3.14). In the control and SC-560 treated animals, the average size of tumors increased by more than 300% relative to baseline (p<0.001), in contrast to 13% reduction in the average tumor size in the celecoxib treated group relative to baseline (p<0.04).

During the six-week treatment period, five new mammary tumors appeared in the control group (a 14% increase, p < 0.05), and there was no spontaneous regression of tumors in control rats (Table 3.14). In contrast, the celecoxib-supplemented diet significantly reduced the overall number of palpable mammary tumors from 27 to 24, an overall decrease of 11% (p < 0.05), as a result of the complete regression of four tumors and the late emergence of one new tumor. Effects of SC-560 on number of tumors were mixed. The regression of four tumors and emergence of one new tumor, a net increase of 7% (p<0.08) was observed. It is notable that tumor regression, partial or complete, occurred in 80% of the celecoxib treated rats, and 50% of the SC-560 treated rats.
Figure 3.11: Treatment effects on the time-course change in mammary tumor volume. The Control group was fed Teklad chow diet; Celecoxib group was fed powdered chow + 1500 mg Celecoxib/kg diet; SC560 group was fed powdered chow + 130 mg SC560/kg diet.
<table>
<thead>
<tr>
<th>Experimental Groups *</th>
<th>Mean Tumor Volume (cm³) b</th>
<th>Total Number of Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>Control</td>
<td>0.33 ± 0.10</td>
<td>1.06 ± 0.25</td>
</tr>
<tr>
<td>SC 560</td>
<td>0.36 ± 0.11</td>
<td>1.10 ± 0.48</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>0.32 ± 0.08</td>
<td>0.28 ± 0.08</td>
</tr>
</tbody>
</table>

Table 3.14: Effects of selective COX-1 and COX-2 inhibitors on growth of DMBA-induced rat mammary tumors.

* Ten rats with established tumors/group were fed powdered Teklad rodent diet, supplemented with 130 mg and 1500 mg/kg diet of SC 560 and Celecoxib, respectively. The data represent 6 weeks of SC 560 and celecoxib therapy.

b Value = Mean ± SEM.

c The divergent trend over time in mean tumor volume between the experimental groups: p < 0.01

d The divergent trend over time in tumor numbers between the experimental groups: p < 0.05

e Change from baseline: p < 0.05

f Change from baseline: p < 0.08

* The number include 5 new tumors and 3 completely regressed tumors during the 6 weeks treatment period.

** The number include one new tumors and 4 completely regressed tumors during the 6 weeks treatment period.
<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Body Weights (gram) *</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial*</td>
<td>Final**</td>
</tr>
<tr>
<td>Control</td>
<td>270.01 ± 6.33</td>
<td>275.50 ± 6.63</td>
</tr>
<tr>
<td>SC 560</td>
<td>267.27 ± 6.73</td>
<td>271.07 ± 5.20</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>266.07 ± 4.30</td>
<td>278.58 ± 7.74</td>
</tr>
</tbody>
</table>

Table 3.15: Effects of selective COX-1 and COX-2 inhibitors on body weights.

* Value = Mean ± SEM

* Difference between the three groups weights : p < 0.89

** Difference between the three groups weights : p < 0.72
**Histopathological Data:** Molecular studies (BrdU and COX-2 staining of mammary cancer tissues) performed on samples from this investigation have indicated, in general, that COX-2 positive nests appears to have higher proliferative indices.

**General Observations:** Weight gain and food consumption were monitored throughout the experiment. The celecoxib and SC-560 supplemented diets did not significantly affect the body weights of the rats. On average, all the treatment groups gained approximately the same weight (Table 3.15). Food intake was almost similar in the three groups. Also SC-560 or celecoxib did not produce clinical or gross changes in several examined organs of the rats that would indicate toxicity.

The overall results of this series of preclinical efficacy studies demonstrate for the first time *in vivo* that the administration of a COX-2 inhibitor (celecoxib), either as chemopreventive or as therapeutic agent, significantly inhibit mammary cancer development and growth in this animal model, and these inhibitory effects are more potent than the non selective COX inhibitor, ibuprofen, or the selective COX-1 inhibitor SC-560.
CHAPTER 4

DISCUSSION

Breast cancer is the most commonly diagnosed malignancy in American females, and despite intensive cancer control efforts, it remains the second leading cause of cancer deaths among American women (Singletary et al., 1998; Cancer Facts, 2000). Such state of affairs has renewed the interest in cancer prevention in general and prompted intense efforts in research and development of chemopreventive agents that might be more applicable and effective in terms of costs and benefits than other forms of prevention.

The development of breast cancer in women appears to be dependent on complex interactions between multiple and diverse factors which can modulate initiation, promotion and progression of this disease, and on the time of exposure to these influences. Indeed, mammary tumorigenesis is greatly influenced by the developmental history of the gland which may provide windows of opportunity for the development of cancer through exposure to different influences (Harris JR et al., 1992; Yoo et al., 1992; Russo et al., 1992). Thus, factors that modulate proliferation, differentiation and apoptosis in a partially differentiated mammary gland would have a great impact on the mammary carcinogenic process.

The COX isozymes and their metabolic products appear to play a key role in the
development and progression of rodent mammary tumors and human breast tumors (Bennett et al., 1986; Carter et al., 1989; Lee et al., 1992; Noguchi et al., 1995). Recent molecular studies of breast tumors have indicated that COX-2 is inappropriately induced and both COX-2 and COX-1 are upregulated in malignant cells (Parrett et al., 1997; Hwang et al., 1998), and that human and animal mammary tumors produce more prostaglandins than benign tumors or normal breast tissue (Schrey et al., 1995; Lupulescu, 1996).

Of particular importance is the observation that genetic expression of COX-2 in human breast cancer cells is correlated with expression of aromatase mRNA in contiguous tissues, and that a chief prostaglandin, PGE₂, effectively and specifically induces the promoter-II region of the cytochrome P450 aromatase gene (CYP19) (Simpson et al., 1994; Zhao et al., 1996). This paracrine effect of PGE₂, therefore, may potentiate the local biosynthesis of estrogen and provide a critical link between prostaglandin production and deregulation of estrogen biosynthesis in mammary carcinogenesis. Recent in vivo evidence supportive of this effect has been reported, where the CYP19 expression was strongly associated with the sum of COXs expression, and a significant positive correlation between the expressions of COX-2 and CYP19 in human breast cancer was observed (Brueggemeier et al., 1999). Several studies have shown that estrogen has proliferative effects, and was shown to upregulate several growth factors such as TGF-α, EGF, pS₂ as well as c-myc, c-fos and c-jun oncoproteins in breast cancer (Dickson et al., 1995). In addition, agents which reduce estrogen level or activity have been associated with decreased risk of breast cancer (Parker et al., 1996).
The signal transduction pathways and transcription factors that mediate the transcriptional activation of COX-2 gene may provide additional important insights into the roles of COX-2 in carcinogenesis. Although specific signaling pathways for all of the several activators and different conditions that lead to transcriptional activation of COX-2 are not yet completely identified, evidence from several studies have suggested that for growth factors, oncogenes, inflammatory mediator as well as phorbol esters, a number of convergent pathways, similar to cellular responses to mitogens, are likely involved in upregulation of COX-2 expression. These include tyrosine kinase signaling pathways; the mitogen-activated protein kinases (MAPK) cascades; as well as nuclear transcription factors (Smith et al., 2000). Enhanced tyrosine kinase activity was observed in many cancers, including breast cancer (Ottenhoff et al., 1992), and deregulated signaling of intracellular tyrosine kinases can promote carcinogenesis (Levitzki et al., 1995). These signaling pathways are in fact loops of autocrine and paracrine effects, since PGs (e.g. PGE₂) were shown to stimulate the production of growth promoting factors such as endothelin, hepatocyte growth factor, estrogen, and stimulatory cytokines which would in turn enhance the production of PGs (Patel et al., 1996; Zhao et al., 1996; Subbaramaiah et al., 1997).

The observations that hormone-independent breast cancer cell lines (which exhibit invasive phenotype) possess an activated c-Ki-ras gene; that ras-transformed cells express elevated COX-2 activity and basal PGE₂ production; and that ras oncoprotein upregulates COX-2 expression in transformed mammary epithelial cells (via both cAMP response element and c-jun/AP-1 activation) have suggested an important role for this oncogene in upregulation of COX-2 expression and PGE₂ production in breast cancer (Schrey et al., 1997).
It has been proposed that the activation of a member of the cytoplasmic kinases such as ras may be one mechanism whereby growth factors stimulate COX-2 expression (Xie & Herschman, 1995). Since estrogen was shown to stimulate the (ER+) mammary cells to produce growth factors (Russo et al., 2001), it is likely that in the undifferentiated or partially differentiated epithelial stem cells, including (ER-) cells, the agonists (e.g. growth factors) activation of tyrosine kinase receptors may lead to activation of ras oncoprotein and one or more of the MAP kinase cascades and downstream activation of c-Jun transcription factor, resulting in transcriptional activation of COX-2 expression. Alternatively, agonists such as fatty acids may activate the PKC pathway, resulting (by activation of the c-Jun transcription factor) in transcriptional activation of COX-2 expression. On the other hand, the reported inhibition of COX-2 expression by normal p53 may explain the observed undetectable levels of COX-2 in normal cells which are increased in many tumors (Subbaramaiah et al., 1999). Thus, the conversion of the normally silent COX-2 gene to a heightened state of constitutive activity observed in cancerous mammary tissue would result in excessive production of prostanoids including PGE\(_2\) and potentiate local estrogen biosynthesis by aromatase.

The aberrant turning on of COX-2 could, therefore, result in several effects on the processes implicated in mammary carcinogenesis, such as mutagenesis by creation of free radical molecules involved in sustained prostaglandin synthesis or metabolic activation of carcinogens such as polycyclic aromatic hydrocarbons; mitogenesis without natural apoptosis due to production of growth factors, estrogen production from aromatase, and production of anti apoptotic factors; angiogenesis by stimulation of neovascularization growth factors’
production; and increased *malignant transformation potential* by modulating the factors that control cell differentiation, invasiveness and metastasis. All or part of these mechanisms may act in concert and explain the link between COX overexpression and mammary tumor growth (Harris RE *et al.*, 1999). The proposal that COX/PGs contribute to mammary carcinogenesis is further supported by epidemiologic evidence that inhibitors of COXs (and thereby of PGs formation) may protect against breast cancer (Schreinemachers *et al.*, 1994; Harris RE *et al.*, 1995; 1996; Subbaramaiah *et al.*, 1997; Harris & Namboodiri, 1999). Therefore, one potential way for breast cancer chemoprevention and/or therapy would be the suppression of COX and PGs production by NSAIDs, particularly the selective COX-2 inhibitors.

The aims of this series of pre-clinical efficacy investigations were to evaluate the chemopreventive and therapeutic effects of NSAIDs in general and selective COX-2 inhibition in particular, against the development and growth of chemically (DMBA)-induced mammary cancer. Evaluations of the optimal dose of COX-2 inhibitor and which stage of the multistep carcinogenic process such agent might exert its maximal effects, were also important aims. In addition, the histological, pharmacological and molecular studies on samples collected from these experiments may help to elucidate, on the cellular and molecular levels, mechanisms proposed to be involved in the anticancer effects of NSAIDs. Another aim of these investigations was to identify an NSAID with lower toxicity and increased efficacy against breast cancer. Since prolonged administration of the common NSAIDs has been associated with gastrointestinal and renal toxicities and many of these NSAIDs have minimal or no selectivity against COX-1 or COX-2, the issues of chronic
tolerability must therefore be carefully considered for prolonged use of such drugs as
chemopreventive or chemotherapeutic agents. One way of minimizing the side effects
(particularly of the GI tract) of prolonged NSAIDs usage is to employ the newly developed
class of selective NSAIDs, COX-2 inhibitors, which have exhibited less toxicity, probably
due to their weaker activity against COX-1.

The results of our investigations are the first to show dramatic suppression of
mammary carcinogenesis in this animal model by COX-2 inhibition. It is also worthy to note
that administration of COX-2 inhibitor, celecoxib, at such doses used, did not produce toxic
side effects such as weight loss, gastrointestinal ulceration, or bleeding.

The precise roles of COXs in general and COX-2 in particular in tumorigenesis in vivo are not yet completely known. Multiple lines of evidence have suggested a link between
level of COXs, particularly chronic activation of COX-2, and tumorigenesis in epithelial
tissues (Sawaoka et al., 1998; DuBois et al., 1998). COX-2 is upregulated in human cancers
of the colon, stomach, and breast (Eberhart et al., 1994; Kargman et al., 1995; Sano et al.,
1995; Ristimaki et al., 1997; Parrett et al., 1997). Animal experiments have also indicated
that induction of COX-2 is a very early event in the sequence of polyp formation and
progression in colon carcinogenesis (Oshima et al., 1996). Increased levels of COX-2 were
found to be present not only in colon tumors but also in apparently tumor-free colonic
mucosa as early as one week after carcinogen administration; and the induction of COX-2
was reported to be an early event in the sequence of polyp formation in colon carcinogenesis
(Singh et al., 1997; Oshima et al., 1996).

Increased levels of COX-2 are also detected in premalignant intestinal tumors in
humans, and in experimental animals (Eberhart et al., 1994; Williams et al., 1996). COX-2 is also detected in premalignant lesions of the head and neck (leukoplakia) and lung (atypical adenomatous hyperplasia) (Wolfe et al., 1998). It has also been reported that forced expression of COX-2 in rat intestinal cells have produced an increased level of bcl-2 protein and increases resistance of the cells to apoptosis (Tsujii et al., 1995). Knockout of the COX-2 gene led to a marked reduction in the number and size of polyps in ApcΔ716 mice (Oshima et al., 1996), and COX-1 and COX-2 knockout mice developed fewer skin papillomas than controls (Tiano et al., 1997).

On the other hand, several epidemiological studies have demonstrated that the risk of colon cancer is significantly lowered by regular and chronic use of NSAIDs such as aspirin, sulindac, and indomethacin (Kune et al., 1988; Rosenberg et al., 1991; 1995; Thun et al., 1991; 1995; Gridley et al., 1993; Greenburg et al., 1993; Schreinemachers et al., 1994; Heath et al., 1994; Giovannucci et al., 1994; 1995; Muscat et al., 1995). In addition, experimental in vitro and in vivo animal studies have shown that synthetic and natural inhibitors of COXs including NSAIDs are potent chemopreventive agents that inhibit and protect against tumor development in rodent models of oral, esophageal, forestomach, colon, bladder, skin, and lung (Cornwall et al., 1983; Reddy et al., 1992; Kellogg et al., 1994; Steele et al., 1994; Mitchell et al., 1994; Huang et al., 1994; Funkhouser et al., 1995; Lupulescu, 1996; DuBois et al., 1996; Duperrone et al., 1997; Castonguay et al., 1993; 1997; Kawamori et al., 1998; Castelo et al., 2000). Furthermore, the use of selective COX-2 inhibitors significantly suppressed polyp formation in mice, and formation of aberrant crypt foci in the intestine of rats (Oshima et al., 1996; Reddy et al., 1996). It was also reported that NSAIDs
suppress not only cancers of epithelial origin but also tumors of mesenchymal origin such as sarcomas and mast cell tumors (Taketo, 1998).

The chemopreventive potential of NSAIDs in breast cancer was not extensively investigated as much. Although early experimental studies in vitro and in vivo on COX inhibition have suggested that NSAIDs possess chemopreventive properties, in the absence and presence of high fat diets to augment mammary tumor growth (Carter et al., 1983; McCormick et al., 1985; Ip et al., 1989; Lee et al., 1992), some epidemiological studies have revealed no statistically significant association between regular use of aspirin and the risk of breast cancer (Paganini et al., 1989; Egan et al., 1991; Rosenberg et al., 1995). However, a renewed interest in the use of NSAIDs for the control of this malignancy has come from recent epidemiological studies which suggest the presence of a significant inverse association between regular intake of NSAIDs and the relative risk of breast cancer (Gridley et al., 1993; Schreinemachers et al., 1994; Harris RE et al., 1995; 1996; Harris & Namboodiri, 1999). The observed statistically significant reduction in the risk of breast cancer incidence was associated with the chronic use of any NSAID regularly, three or more times per week, for at least a year. This protection was found to be similar for users of aspirin alone, ibuprofen alone, and all NSAIDs combined (Harris & Namboodiri, 1999).

The nonselective NSAID, ibuprofen, has activity against COX-1 and COX-2 isozymes but relatively low COX-2 inhibition compared to COX-1 (Smith & DeWitt, 1996). The results of our investigations of the chemopreventive potential of ibuprofen against the DMBA-induced mammary tumors in female Sprague-Dawley rats have revealed that ibuprofen has potent, dose-dependent chemopreventive effects, in terms of inhibition of
incidence, tumor burden (multiplicity) as well as reduction in tumor volume, that are comparable to those of the potent chemopreventive retinoid, 4-HPR, which suggest that ibuprofen may have a chemopreventive potential against breast cancer similar to that observed by others for colorectal cancer. Extensive in vitro and in vivo studies have demonstrated the chemopreventive and chemotherapeutic efficacies of the retinoid, N-(4-hydroxyphenyl) retinamide (4-HPR), alone or in combination with other chemopreventive agents, against breast cancer in this model system (Curley et al., 1996; Abou-Issa et al., 1997; Bhatnagar et al., 1991). Molecular studies on samples from these investigations have shown that gene expression of both COX-1 and -2 was inhibited by ibuprofen and 4-HPR, while localization studies of COXs immunoreactive proteins have indicated more localization of COX-1 immunospecific protein to the inflammatory cells in the stroma, and more localization of COX-2 immunospecific protein within tumor cells.

The commonly used nonspecific NSAIDs such as ibuprofen are known to inhibit the activity of both COX isozymes which probably account for both their therapeutic and adverse side effects that have limited their wide use as chemopreventive agents. The discovery of COX-2 isoform, illustrating its structure, and identifying the pathological functions attributed to its activity have led to the development of its specific inhibitors. The effects of COX-2 blockers are attributed not only to greater activity against this isoform, but also to weaker activity against COX-1 (Ramesha et al., 1997).

Extending our investigations to the chemopreventive effects of a selective COX-2 inhibitor, celecoxib, in the same animal mammary cancer model, have revealed that daily administration of this specific COX-2 inhibitor has remarkably suppressed the incidence,
multiplicity, and size of malignant breast tumors induced by the carcinogen DMBA. The observed chemopreventive effects of the specific COX-2 inhibitor, celecoxib, exceeded those of the general NSAID, ibuprofen, further supporting the proposal that COX-2 is a key culprit in carcinogenesis. It also exceeded the obtained effects of the retinoid, 4-HPR, which has shown significant chemopreventive efficacy in this animal model (Abou-Issa et al., 1995).

The observed higher potency of COX-2 inhibitor, celecoxib, in vivo suggests that these agents may have a chemopreventive advantage over the nonspecific NSAIDs that inhibit both COX-1 and COX-2 isoforms, and underscores the potential usefulness of these low toxicity agents in cancer chemoprevention for individuals at high risk for breast cancer development, and under conditions where other chemopreventive agents may be contraindicated.

It is worthy to reiterate that the chemopreventive effects of COX-2 inhibitors have been observed against other types of malignancies, the most notable of which are colon and bladder cancers (Sheng et al., 1997; Kawamori et al., 1998; Reddy et al., 2000; Grubbs et al., 2000), thus, supporting the possibility that COX-2 blockers may have a value as a general chemopreventive agent against a spectrum of malignancies.

The observed greater COX-2 inhibitor’s ability to reduce tumor formation does not necessarily explain the apparent sensitivity of the tumor tissues. Basically, the in vivo anticarcinogenic effects of an agent will likely depend on the local concentration of the agent (local bioavailability) as well as certain other factors in the incipient tumor micro-environment. Taken together, the observations of celecoxib’s high distribution in body tissues, the COX-2/PGs elevation in tumor tissue, the mammary gland high content of fatty
tissue, and the link between elevated COX/PGs and increased local estrogen biosynthesis, may explain the sensitivity of mammary tumors to COX-2 inhibition.

The experience with chemopreventive agents have indicated that they act in a dose-dependent manner, along with their associated toxicities. Since a main premiss of chemoprevention is chronic usage of such agent(s), examining the relative chemopreventive effect of varying doses of COX-2 blocking agent (celecoxib) on mammary carcinogenesis in order to identify the lowest possible dose with optimal efficacy is of great importance. Also baseline information for the application of such agents in future clinical investigations may be required. Our results indicate that the administration of increasing doses of the COX-2 blocking agent (celecoxib) have significantly inhibited mammary tumor incidence and multiplicity as well as tumor volume in a dose-dependent manner. There was a concomitant increase in the steady-state serum concentration of the drug with the dose. The positive correlation between serum levels and the extent of inhibition of tumor incidence was highly significant. The difference in inhibition of mammary cancer incidence and multiplicity between a lower dose (500 ppm) and the highest dose (1500 ppm) was rather limited, indicating that, in this preclinical model, the chemopreventive action against breast cancer of COX-2 blockade is effective even at lower dose levels. It is worthy to note that exposure to the drug in rats receiving the lower dose (500 ppm) is approximately equivalent to that achieved in human females given the drug at a 200 mg twice daily (McEvoy et al., II, 2000). This observation is of great interest as it demonstrates that even low doses of COX-2 inhibitors, can be used as effective chemopreventive agents against breast cancer. Molecular studies on samples from these investigations have shown that COX-2 expression was
detected in both the malignant mammary epithelial cells as well as in the inflammatory cells and vasculature associated with the neoplastic lesions in this rat mammary tumor model. This observation suggests that COX-2 may play a functional role in tumor-induced angiogenesis, and that suppression of COX and prostaglandins in the vasculature by COX-2 inhibitors may block neovascularization, and hence tumor growth.

The multistage carcinogenesis generally involves interactions between three main types of control systems: the control of gene expression brought about by agonist-induced signals transduction; cell cycle control; and control of the fidelity of DNA and chromosome replication. All of these systems interact within themselves and between each others, which may be done through complex cascades of proteins activation/deactivation, and cascades of genes expression. Exogenous (both genotoxic and non genotoxic) and endogenous (both inherited and acquired) factors can affect all three systems and drive the multistages carcinogenic process. Several studies have suggested possible mechanisms by which COXs, particularly COX-2, might influence the different phases of carcinogenesis. Such observed effects, which can be reduced by COX-2 inhibition, are likely to increase the carcinogenic potential of affected altered cells and favor their growth.

The formation of free radicals has been linked with the metabolism and oxidation of prostaglandins (Marnett, 1994). In tobacco-related malignancies, it has been reported that the peroxidase activity of COX can activate a broad array of xenobiotics, including polycyclic aromatic hydrocarbons and tobacco-specific nitrosamines, to reactive metabolites which can affect DNA and proteins (Rioux et al., 1998). The persistent presence in tumor tissue of COX-2 (and consequently high levels of PGs), which is constitutively expressed and
upregulated in a number of human tumors including breast cancer, may result in increased expression of genes normally induced only transiently during passage through the cell cycle (Han et al., 1990; Tsujii et al., 1995). Enhanced expression of COX-2 and synthesis of PGs, particularly PGE$_2$ and PGF$_{2\alpha}$, is associated with inflammation, cell proliferation, and angiogenesis (Marnett, 1992; O’Neill et al., 1993; Eberhart et al., 1994; Herschman et al., 1994; DuBois & Awad, 1994; Lupulescu, 1996; Masferrer et al., 2000). The pronounced expression of COX-2 in human neoplastic epithelium, as well as associated neovasculature support the proposal that COX-2 inhibition in human cancer would inhibit the production of angiogenic growth factors by tumor cells (Tsujii et al., 1998; Masferrer et al., 2000).

Several studies have linked the growth and invasive potential of human tumors, including mammary tumors with increased production of prostaglandins such as PGE$_2$ and PGF$_{2\alpha}$ (Rolland et al., 1980; Honn, 1981; Marnett, 1992; Liu XH et al., 1996; Liu et al., 1996; Tsujii et al., 1997; Taketo, 1998). Elevated PGE$_2$ level is a characteristic of hormone-independent breast tumors with a high metastatic potential, and (ER-) cell lines which exhibit an invasive phenotype were reported to have a high capacity for PGE$_2$ production following stimulation with IL-1β or activation of PKC pathway (Boorne et al., 1998). In addition, PGs such as PGE$_2$ and PGF$_{2\alpha}$ are potentially important throughout the carcinogenic process through their modulation of immune surveillance, and several signal transduction pathways (Marnett, 1992; Jones et al., 1993; DuBois & Tsujii, 1994; Hla et al., 1997; Shiff et al., 1999). The overexpression of COX genes and increased PGE$_2$ in epithelial cells were also shown to increase the expression of the anti-apoptotic oncoprotein Bcl-2 and to inhibit programmed cell death (apoptosis) (Tsujii et al., 1995; Piazza et al., 1995; Lu X et al., 1995;
Siûf et al., 1995). Higher level of COX-2 was also observed in human glioma than normal brain specimen, and a selective COX-2 inhibitor was shown to reduce in vitro the proliferation as well as migration of the tumor cells (Joki et al., 2000). A selective COX-2 inhibitor was also reported to cause anti-angiogenic action, suppression of tumor proliferation through cell cycle arrest, and inhibition of telomerase activity in human head and neck squamous carcinoma cells (Nishimura et al., 1999).

The proposed multiphase nature of carcinogenesis also provides opportunities for intervention with agents targeted at specific mechanisms assumed to be involved in the initiation, promotion and progression phases of cancer. One of our investigations has attempted to distinguish between the differential effects of COX-2 inhibition on the initiation and promotion/progression phases of mammary carcinogenesis, in other words, to determine the independent effects of this agent (celecoxib) during the Initiation (I), Promotion (P) or Initiation + Promotion (I+P) phases of chemically (DMBA)-induced rat mammary carcinogenesis. The obtained results support the proposal that COX-2 may play a key role in both the initiation and promotion/progression phases of breast cancer. With COX-2 inhibitor, administered during the I, or P phases, the tumor incidence, tumor burden (multiplicity), and tumor volume were similarly and significantly inhibited as compared to the control group. The sustained presence of COX-2 inhibitor during the initiation + promotion (P+I) phase produced the maximal inhibition of mammary tumor development and growth. These results are the first to show that COX-2 inhibitor (celecoxib) acts on (I) and (P) phases almost to the same extent, with the effect being maximal when given continuously during the whole I+P phase.
In regard to the chemotherapeutic studies, the antineoplastic (chemotherapeutic) drugs can be conveniently categorized into two broad categories: cytotoxic and antiproliferative groups. The mechanisms of action of antiproliferative category members are varied, and many of these mechanisms are still unknown. They are reported to exert various effects such as apoptosis; cell-cycle-phase related effects such as placing cells at rest or inhibiting mitosis; anti angiogenesis effects; or hormonal effect modulators; among many other putative mechanisms. The evidence suggests that NSAIDs act as an antiproliferative agents (Hughes-Fulford et al., 1997).

It is proposed that the mammary cancer progression yield could be measured by the Cancer Load (CL) which can be defined in terms of the size (volume) and number (primary and secondary) of the cancerous lesions. Therefore, the antineoplastic (chemotherapeutic) effects of an agents can be measured in terms of its inhibitory effects on the growth of tumor volume and numbers.

As discussed earlier, COXs and their products appear to play different important roles in cancer progression, which provide a rationale for incorporation of COX-2 inhibitors, particularly with the low gastrointestinal adverse side effects associated with them, into the treatment regimens of different clinically detected cancers. Epidemiological studies have shown that NSAIDs can reduce the size and number of colorectal cancer lesions in Familial Adenomatous Polyposis (FAP) patients (Thun et al., 1991; 1995). Clinical studies in patients with FAP, who are considerably predisposed to colon cancer, have also indicated that administration of the NSAID, sulindac, caused marked regression in adenomas size and number, and prevented the recurrence of adenomatous polyps, considered as precursors for
colon cancer (Giardiello et al., 1993; Spagnesi et al., 1994). As chemotherapeutic agents
NSAIDs such as ibuprofen and piroxicam have been shown to induce tumor regression in
animal models of colon carcinogenesis, as well as papillomas in the mouse lung and fore­
stomach (Kelloff et al., 1994; Pereira et al., 1996), and the NSAID sulindac has caused a
decrease in the number and size of polyps in Min mice (Boolbol et al., 1996). Also
experimental in vivo studies have shown that a selective COX-2 Inhibitor, reduced the
number and size of the intestinal polyps in ApcΔ716 knockout mice (Oshima et al., 1996).

Again, the potential of NSAIDs in the therapy of breast cancer was not much
investigated. Establishing the antineoplastic efficacy of COXs, particularly COX-2 inhibition
against chemically induced animal mammary cancer may support their incorporation in
future human clinical trials as an adjuvant chemotherapeutic agent against breast cancer.
Therefore, an aim of our preclinical efficacy studies was to determine whether COX-2
inhibitor (celecoxib) has antineoplastic effects on the cancer load (CL) of pre-established
DMBA-induced rat mammary tumors. Our investigation with the nonselective NSAID
(ibuprofen) have revealed antineoplastic effects in terms of reductions in tumor size in this
chemically-induced animal mammary model. The average tumor volume for the ibuprofen
treated animals at the end of the treatment period was markedly lower than that of controls
whose tumors continued to grow actively. Consistent with these effects, the growth arrest
was associated with inhibition of the genetic expression of both COX isoforms.

Extending the investigation into the antineoplastic (therapeutic) efficacy of specific
COX-2 inhibition have revealed significant growth inhibitory effects of the COX-2 inhibitor
(celecoxib) on the volume as well as the number of established DMBA-induced mammary
tumors, vis-a-vis cancer load. The average tumor volume for the celecoxib-treated animals at the end of the treatment period was markedly lower than that of controls, and was moderately reduced from baseline. Also the number of tumors for the treated group was significantly lower than that at baseline, whereas tumors continued to emerge in the control group. This is the first report on the regression of mammary tumors in vivo by a COX-2 inhibitor. It is also notable that tumor regression occurred in 90% of the celecoxib treated rats, which may be relevant to humans in whom a drug is considered active in any particular tumor histotype if it gives positive results in at least 70% of the patients (Stofli et al., 1988).

The role of COX-1 in carcinogenesis, including mammary carcinogenesis, is not yet clarified. COX-1 was reported to be expressed in both normal and neoplastic regions in many tissues, including mammary tissue, but seems to be particularly expressed in the tumor stroma that include fibroblasts, smooth muscle cells, and the vasculature; however, the observed broad distribution of this isoform in both normal and neoplastic tissues makes it difficult to determine its exact role in carcinogenesis (Masferrer et al., 2000). Studies of the anticarcinogenic effects of selective COX-1 inhibitors are also scarce. Initial experimental studies with resveratrol (a phenolic antioxidant phytoalexin found in grapes and other foods), an agent initially assumed to be a specific COX-1 inhibitor, have shown that it has anti-inflammatory and anticarcinogenic effects in mice mammary preneoplastic lesions and mice skin cancer model. However, it was reported later that the effects of resveratrol are predominantly through the inhibition of COX-2 activity and gene expression (Subbaramaiah et al., 1998). Interestingly, resveratrol was reported to be a phyto-estrogen that stimulates the growth of estrogen-dependent breast cancer cells (Subbaramaiah et al., 1998).
Since the antineoplastic (therapeutic) efficacy in breast cancer of COX-1 inhibition (with synthetic selective COX-1 inhibitor) has not been compared in vivo against COX-2 inhibition (with synthetic selective COX-2 inhibitor), an investigation to compare in vivo the relative effects of COX-1 versus COX-2 inhibition on progression of established DMBA-induced mammary tumors was performed, and it is the first to indicate that COX-2 is the predominant isozyme in tumor progression.

The observed antineoplastic effects of COX-1 inhibitor (SC560) and COX-2 inhibitor (celecoxib) treatment on cancer load (CL) were markedly different. The average tumor volume of COX-1 inhibitor (SC560)-treated animals at the end of the treatment period was almost the same as that of controls, and both were much higher than their initial measurements. In contrast, the average tumor volume for the COX-2 (celecoxib)-treated animals at the end of the same period was markedly lower and was moderately reduced from baseline. The number of tumors significantly increased in the control animals, in contrast to their decreasing number in the COX-2 inhibitor (celecoxib)-treated animals over the same time period. The COX-1 inhibitor (SC560)-treated animals have also shown a statistically non significant increase in tumor number. It is also notable that tumor regression, partial or complete, occurred in 80% of the COX-2 (celecoxib)-treated animals. Selective COX-2 blockade therefore appears to affect growth arrest of malignant breast lesions in this animal model, whereas selective COX-1 blockade has no effect. These results provide further evidence that selective COX-2 inhibition may have a significant role in breast cancer therapy, as the higher potency of selective COX-2 inhibition may provide a chemotherapeutic advantage over other nonselective NSAIDs.
The overall results of these preclinical efficacy studies support the epidemiologic findings suggesting that NSAIDs may have chemopreventive/therapeutic value against breast cancer. They also indicate that selective COX-2 inhibition has a potent antineoplastic (chemotherapeutic) effects, in addition to its anticarcinogenic (chemopreventive) effects, and underscore the need for more intensive investigation of COX-2 inhibition, as a potentially effective approach to the prevention and therapy of this disease. The higher potency observed and the reported lower toxicity of COX-2 inhibitors may provide chemopreventive and chemotherapeutic advantages over other nonselective NSAIDs, and suggest that it is feasible to recommend the use of COX-2 inhibitors as chemopreventive and/or therapeutic agents against breast cancer in high risk individuals.

The exact mechanisms of action by which COX-2 blockers and other NSAIDs inhibit carcinogenesis, including mammary cancer remain to be clarified. Traditionally, the anti-inflammatory, anti-pyretic and anti-nociceptive activities of NSAIDs have been explained by its COX-mediated inhibition of prostaglandin synthesis. Thus, lacking sufficient information to formulate a mechanism-based classification of NSAIDs' anticarcinogenic effects, the currently used classification, derived essentially from the pharmacological studies of NSAIDs effects and based apparently upon the inhibition of cyclooxygenase catalysis and PG synthesis, categorizes the proposed mechanisms as COX/PGs-dependent or COX/PGs-independent mechanisms (Shiff et al., 1999).

Some difficulties regarding such classification may arise from limiting the focus on the catalytic activity of one site of COX enzyme and not considering the inhibition of the enzyme gene expression, which may leave some of the NSAIDs' effects unaccounted for.
The COXs can promote carcinogenesis, aside from producing several prostanoids, by producing free radicals or activating carcinogens via its peroxidase activity (Marnett, 1994), and the cyclooxygenase activity of both COX-1 and COX-2 can be inhibited by most NSAIDs, which compete directly with arachidonate for binding to the cyclooxygenase active site, however, NSAIDs in general have little or no effect on the peroxidase activity (DeWitt, 1999).

In addition, although the pharmacological bases for the effects of NSAIDs in general appear to be related primarily to their inhibitory effects on COX isozymes’ cyclooxygenase catalytic activity (with subsequent reduction in prostaglandins’ production) (Subbaramaiah et al., 1996; Shiff et al., 1999), the inhibition of COX isoforms gene expression (and mechanisms proposed to mediate such inhibition) by some NSAIDs and other COX inhibitors have also been reported (Boolbol et al., 1996; Tjandrawinata et al., 1997; Gallois et al., 1998; Schwenger et al., 1998; Subbaramaiah et al., 1998; Tegeder et al., 2000). Our investigations with the nonselective NSAID, ibuprofen have also indicated inhibition of both COX-1 and -2 isoforms gene expression. Thus, the characterization of antitumor mechanism as COX-dependent mechanism in this context should include inhibition of COX gene expression. It is interesting to note that, with all the data on the structure and regulation of COXs, there is no systematic investigation yet to elucidate the regulatory effects of NSAIDs on COX gene expression, and the links of these regulatory mechanisms with the modulation of other genes’ expression and signaling pathways in vivo. This is of importance since some of the mechanisms that are considered COX/PGs-independent are also associated with regulation of COX gene expression and subsequent PG production. For example, some
NSAIDs such as sodium salicylate and R-flurbiprofen, which were shown not to inhibit the cyclooxygenase catalytic activity in vitro, were reported to exert their anti-inflammatory effects through inhibition of transcription factors such as NF-κB and AP-1 activities (mechanisms that may be classified as COX-independent), the same mechanisms by which they were reported to block COX-2 gene expression (and lower PGs levels) during these studies (Schwenger et al., 1998; Gallois et al., 1998; Tegeder et al., 2000).

Another issue that contribute to the observed uncertainty in the literature regarding the classification of a mechanism as COX/PGs-dependent or COX/PGs-independent arise from the observations that some of the mechanisms proposed, such as apoptosis, angiogenesis or tumor-related immunity, are associated with both COX/PGs-dependent and -independent processes (Shiff et al., 1999; Nishimura et al., 1999). Nevertheless, some COX-independent mechanisms, which have also been demonstrated to be active in COX null model systems, may account for at least some of the proposed anticarcinogenic effects of NSAIDs (Shiff et al., 1999, He et al., 1999).

Since the well documented pharmacological actions of NSAIDs are known to be inhibition of COXs' cyclooxygenase catalytic activity (thereby reducing PGs production), which is thought to be the predominant mechanism by which they act as analgesic, antipyretic, and anti-inflammatory agents, and given the multiple lines of evidence suggesting a link between elevated levels of COX-2 (and PGs) and tumorigenesis in several tissues, including epithelial tissues, it can be inferred that the beneficial chemopreventive/therapeutic effects of NSAIDs may be mediated predominantly through the inhibition of prostaglandin (PG) biosynthesis (Mitchell et al., 1994; Hughes-Fulford et al., 1997).
As amply mentioned above, evidence that COX isozymes (and PGs) are important in carcinogenesis include the observed COX-2 upregulation and PGs (particularly PGE₂) levels' elevation in cancerous more than normal or benign tissues; the observed PGs' stimulation of proliferation and resistance to apoptosis in different cell types, and the demonstration of COX-2 contribution to carcinogenesis in knockout mice (Oshima et al., 1996; Sheng & Shao, 1998; Shiff et al., 1996; Sawaoka et al., 1998). In addition to producing several PGs, COX isozymes may enhance carcinogenesis either by activating carcinogens via its peroxidase activity (which can operate on substrates other than PGG₂), or by producing peroxy radicals or malondialdehyde (MDA) which is a direct acting mutagen (Marnett, 1994). The COX-2 and PGE₂ were reported to induce angiogenesis, in part through the induction of vascular endothelial growth factors (Ben-Av et al., 1995; Tsujii et al., 1998), and NSAIDs to inhibit angiogenesis (Skopinska et al., 1998). Over expression of COX isozymes in human breast cancer cells was shown to activate metalloproteinase-2, involved in cancer cells mobility, invasiveness, and metastasis (Takahashi et al., 1999), and NSAIDs were shown to inhibit it (Lempinen et al., 2000). While several reports describing effects on cultured cells have suggested an antiproliferative effect, involving cell cycle arrest, as responsible for the growth inhibitory activity of NSAIDs, other studies have reported NSAIDs to promote apoptosis in transformed and cancer cells, and the protective effect of NSAIDs may thus be explained by the induction of apoptosis (Lu et al., 1995; Pasricha et al., 1995; Piazza et al., 1995; Boolbol et al., 1996; Sawaoka et al., 1998). NSAIDs may enhance the mechanisms of tumor immune surveillance, as it was shown that PGE₂ reduces the transcription of MHC-II (major histocompatibility complex, class -II) antigen molecules.
and NSAIDs can upregulate it (Arvind et al., 1995). PGE₂ was also shown to inhibit the production of immune regulatory lymphokines; to inhibit T and B-cell proliferation, and the cytotoxic activity of natural killer cells; to inhibit the production of tumor necrosis factor, while induce the production of IL-10 which has immuno-suppressive effects; and to inhibit monocytes' potential to migrate in the direction of a chemotactic stimulus and to adhere to endothelial cells, probably due to modulation of receptor and adhesion molecules (Subbaramaiah et al., 1997; Zeidler et al., 2000). PGI₂ was also reported to block leukocyte adhesion to endothelial cells and to inhibit monocyte activation (Wu, 1996). In relation to breast cancer, inhibition of COX-2 and PGE₂ may reduce expression of the cytochrome P-450 aromatase gene (CYP-19), thus preventing local synthesis of estrogen and removing a major stimulus of breast cancer growth (Simpson et al., 1994; Zhao et al., 1994; Lu X et al., 1995; Dickson et al., 1995).

Some NSAIDs were reported to suppress PPARδ activity and promote apoptosis (He et al., 1999), however, the link with the transcription factors PPARs is somewhat complicated. PPARδ was shown to be associated with colon cancer, and NSAIDs such as sulindac sulfide and indomethacin were shown to inhibit PPARδ activity, through both inhibition of PGs (PGI₂ was reported to be ligand for PPARδ and stimulate its RXRα dimer binding to the target gene response element), and also independently (directly) through disruption of binding of the PPAR/RXR transcription complex (He et al., 1999). On the other hand, some NSAIDs such as indomethacin have the ability to activate PPARα and PPARγ (Lehmann et al., 1997), which are generally associated with differentiation of fibroblasts to adipocytes, and are also associated with the proposed inhibitory cyclopentenone PGs such
as PGJ₂ derivatives (Kliwe et al., 1995). However, although activation of PPARγ in colon
cancer cells in vitro was shown to reduce their growth and increase their differentiation
(Sarak et al., 1998), PPARγ activation was reported to increase colon tumorigenicity in
APCmin mice (Lefebvre et al., 1998).

Thus, the COX-dependent mechanisms that can be influenced by NSAIDs and
contribute to their anticarcinogenic effects may include: carcinogen formation, angiogenesis,
cell proliferation, apoptosis, tumor-related immunity, and modulation of certain transcription
factors such as PPARs (Shiff et al., 1999). Therefore, inhibitors of COX-2 (and PGs
synthesis) such as NSAIDs, retinoids, and curcumin, may decrease the formation of
mutagens and at the same time enhance processes such as immune surveillance and apoptosis
which tend to destroy initiated cells, thus protect against carcinogenesis (Marnett, 1992;
Huang et al., 1992; Giardiello et al., 1993; Rao & Zang, 1995; Rao et al., 1995;
Subbaramaiah et al., 1996; Kawamori et al., 1999).

However, there are other observations that suggest the presence of additional cellular
targets and are difficult to reconcile with COX (PGs) being the sole target of NSAIDs. For
example, NSAID derivatives which lack the ability to inhibit COX/PGs were reported to
inhibit colonic tumor growth in vivo and in vitro, and to have protective effects against
mammary tumors in animals (Thompson et al., 1997; Reddy et al., 1999). The colon cancer
cells that are totally devoid of COX activity were reported to be growth inhibited by NSAIDs
as effectively as cells producing COX (Elder et al., 1997), and in those cancer cells
producing COX, the COX-derived prostaglandins cannot rescue cells from NSAIDs-
associated growth arrest in vivo or in vitro (Hanif et al., 1996; Chan TA et al., 1998).
NSAIDs have also been reported to suppress the proliferation and cell transformation, and to initiate apoptosis in COX null fibroblasts, as COX-1 and COX-2 null mouse embryo fibroblasts were shown to remain sensitive to the antiproliferative and antineoplastic effects of NSAIDs (Zhang et al., 1999). The concentration of NSAIDs that inhibit growth is often many times higher than that required to inhibit COX activity, suggesting other cellular targets (Simmons et al., 1999). In different experiments with sulindac, indomethacin and carprofen (which all reduce PGs synthesis), the addition of exogenous PGs did not reverse the growth inhibitory effects of NSAIDs (Piazza et al., 1995). Furthermore, the sulfone derivative (which lack the ability to inhibit COX/PGs) of the NSAID sulindac was reported to inhibit colon carcinoma cells growth by inducing apoptosis; to inhibit tumor formation and growth in colon cancer model and induce apoptosis without significantly reducing PGs levels in the stomach and colon; and in NMU-induced mammary tumor model, sulindac sulfone was reported to have potency comparable to sulindac for inhibiting tumor formation (Piazza et al., 1995). In chicken embryo v-src-transformed fibroblasts, several NSAIDs were reported to cause apoptosis, independent of COX, through significant repression of p20, a protein that may protect against apoptosis when fibroblasts enter the G_0 phase (Taketo, 1998). Also NSAIDs were reported to inhibit the cytochrome P-450 that activate carcinogens suspected to cause cancers (O’Brien et al., 1996). Experimental studies in vitro also suggested that the NSAID, ibuprofen, may be a free radical scavenger (Tahamont et al., 1986). It was proposed that NSAIDs, the highly protein bound molecules that probably interact with and inhibit the function of many proteins and other molecules, may inhibit cell proliferation, independently of COX activity, by inducing cell cycle quiescence, in part by reducing the levels of several
key molecules that catalyze the transition through the various phases of the cell division cycle (Shiff et al., 1996; 1999).

The proposed apoptosis model of NSAIDs, arachidonate, and ceramide is somewhat complex, and does not fully explain the apoptosis induced by NSAIDs in COX null cells (Shiff et al., 1999). It was reported that blocking of prostanoid biosynthesis by NSAIDs’ treatment of human mammary epithelial cell and colon cancer cells, have resulted in an increase in intracellular arachidonic acid (reported to be an inducer of apoptosis), which in turn activate the enzyme sphingomyelinase that convert sphingomyelin to ceramide, a known mediator of apoptosis (Subbaramaiah & Dannenberg, 1998; Chan TA et al., 1998), and treatment of colon cancer cells with ceramide caused significant arrest of the cell cycle, and induced apoptosis (Chan TA et al., 1998). Technically, this apoptotic model may be considered as COX (PGs)-independent, however, ceramide, a lipid second messenger, was also shown to induce COX-2 and PGE₂ production in human mammary epithelial cells (Subbaramaiah & Dannenberg, 1998), and sphingomyelinase which releases ceramide from sphingomyelin, also activate COX-2 transcription (Smith et al., 2000), thus the pro-apoptotic effects of ceramide may be counterbalanced by its induction of COX-2 which overexpression was shown to inhibit apoptosis (Subbaramaiah & Dannenberg, 1998).

It is worthy to note, however, that depending on the model and type of cells used, the effects of NSAIDs on the release of arachidonate appear to be variable. For example, in glomerular mesangial cells, aspirin was reported to inhibit (through inhibition of NFκB DNA-biding activity) the expression of mRNA and protein of the IL-1β -inducible group II secretory phospholipase A₂ (sPLA₂) which act upstream of COXs (Vervoordeldonk et al., 2000).
1996). Also, the arachidonate-induced formation of lipid-bodies (non membranous substrate lipid precursors) in leukocytes was shown to be inhibited by aspirin and sodium salicylate, and it seems that this effect on lipid bodies appears to be independent of COX isozymes since it was not observed in macrophages from homogenous COX-1 or COX-2 knockout mice (Bozza et al., 1996). The inhibition of lipid bodies formation by aspirin and sodium salicylate was proposed to be mediated by the inhibition of NF-κB and other transcription factors (Kopp et al., 1994; Ghosh et al., 1995; Bozza et al., 1996).

The transcription factors including NF-κB and AP-1 are essential regulators of the expression of viral oncogenes as well as a number of genes involved in regulation of immune, inflammatory and growth responses (Baeuerle et al., 1996; Gallois et al., 1998; Tegeder et al., 2000). They are also involved in the transcriptional activation of COX-2 (Smith et al., 2000). They are inhibited by several NSAIDs (Gallois et al., 1998; Tegeder et al., 2000), and NSAIDs’ inhibition of NF-κB and AP-1 binding was shown to be independent of the COX cyclooxygenase activity inhibition (Tegeder et al., 2000). Some NSAIDs such as indomethacin, however, do not inhibit NF-κB or AP-1 activities (Shiff et al., 1999).

Several studies have shown that NSAIDs inhibit cell transformation, independently of COX activity, either through inhibition of AP-1 activation or directly inhibiting ras signaling by binding to ras (Hermann et al., 1998), and ras was shown to influence many pathways in cellular transformation. The NSAIDs were also shown to influence the microsatellite instability in cancer cell lines deficient in DNA mismatch repair, which have died by apoptosis in response to NSAIDs treatment (Ruschoff et al., 1998). The NSAID, sulindac (in addition to inhibiting PG production) was reported to significantly block the
formation of some of the lipooxygenase pathway metabolites (HETEs) in the colonic mucosa (Reddy et al., 1996).

Therefore, the COX-independent mechanisms that can be influenced by NSAIDs and contribute to their anticarcinogenic effects may include: ras signal transduction, MAPK kinase activation, angiogenesis, cell proliferation, apoptosis, cell transformation, DNA repair, and modulation of certain transcription factors such as NFkB, and AP-1 (Shiff et al., 1999).

In all likelihood, the NSAIDs, including specific COX-2 inhibitors, may bring about their chemopreventive and therapeutic effects in mammary cancer through both COX-dependent and -independent mechanisms. A challenge will be to determine which of these or other unknown mechanisms produce the remarkable anticancer effects of the COX-2 inhibitor observed in our investigations, as well as the relative contribution of each mechanism.

Conclusion

The results of these pre-clinical efficacy studies underscore the potential usefulness against breast cancer of COX-2 inhibition as a chemoprevention modality, particularly for individuals at high risk of developing breast cancer, as well as a chemotherapeutic modality as an adjuvant therapy to established treatments.
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