12-2001

Beta-Adrenergic, Arachidonic Acid and Potassium Channel Associated-Regulation of Human Breast Cancer Cell Lines

Yavuz Cakir
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Yavuz Cakir entitled "Beta-Adrenergic, Arachidonic Acid and Potassium Channel Associated-Regulation of Human Breast Cancer Cell Lines." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Dr. Hildegard M. Schuller, Major Professor

We have read this dissertation and recommend its acceptance:

Sharon M. Patton, Michael F. McEntee, John L. Bell

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

I am submitting herewith a dissertation written by Yavuz Cakir entitled "Beta-adrenergic, arachidonic acid and potassium channel associated-regulation of human breast cancer cell lines." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Dr. Hildegard M. Schuller
Major Professor

We have read this dissertation
and recommend its acceptance:

Sharon M. Patton

Michael F. McEntee

John L. Bell

Accepted for the Council:

Dr. Anne Mayhew
Vice Provost and
Dean of Graduate Studies

(Original signatures are on file in the Graduate Student Services Office.)

BETA-ADRENERGIC, ARACHIDONIC ACID AND POTASSIUM CHANNEL-ASSOCIATED REGULATION OF HUMAN BREAST CANCER CELL LINES.

A Dissertation
Presented for the
Doctor of Philosophy Degree
The University of Tennessee,
Knoxville

Yavuz Cakir
December, 2001

DEDICATION

This thesis is dedicated to my wife Iklime Cakir as well as my sons, Mehmet Emre and Enes whose patience, supports and encouragements were invaluable to achieve this dream.

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to my adviser Dr. Hildegard Schuller whose advice, support, encouragement and patience made this degree possible. I also wish to thank the members of my committee, Dr. John L. Bell, Dr. Michael McEntee, and Dr. Sharon Patton, for their time and valuable suggestions. In addition, I thank Dr. Howard Plummer for his technical assistance in the completion of my dissertation. I am very grateful for the financial assistance provided by the Comparative and Experimental Medicine in order to finish my dissertation.

Finally, a very special thank is extended to my loving wife, Iklime for her endless tolerance, patience, devotion, and consideration during my studies.

ABSTRACT

During the late stages of breast cancer progression, breast cancer cell growth switches from steroid hormone to growth factor dependence, and it is assumed that invasive breast cancers are growth factor receptor positive and estrogen hormone resistant. The cellular arachidonic acid (AA) pathway is upregulated in a variety of cancer types, and it may play an important role in the growth regulation of breast cancer cells.

Recent evidence suggests that beta-adrenergic receptors (β-ARs) are expressed in some estrogen receptor (ER) negative breast cancers and that beta agonists not only can trigger AA release via the activation of cytosolic PLA_2 or MAPK but also stimulate DNA synthesis in ER (-) breast cancers. AA is metabolized to prostaglandins and leukotrienes by cyclooxygenases (COX-1, 2) and 5-lipoxygenase (5-LOX), respectively. COX-1 is constitutively expressed and required for the homeostatic function, whereas COX-2 is overexpressed during pathological conditions and cancer. It is well documented that COX-2 and 5-LOX are overexpressed in ER (-) breast cancers. Additionally, in many cell types there is a link between G-protein inwardly rectifying potassium channel 1 (GIRK1) and beta-adrenergic receptor pathways. A recent report has demonstrated overexpression of GIRK1 in metastatic breast carcinomas.

Using thymidine incorporation assays, this study shows that antagonists of β-AR, COX, 5-LOX or potassium channels in vitro decreased cell proliferation in both ER (-)

and (+) breast cancer cell lines. The general β-blocker propranolol was more effective than the selective β2-antagonist ICI 118,551 or the selective β1-antagonist atenolol. ER (+) cell lines generally were more responsive to the β1-antagonist atenolol whereas ER (-) cell lines were more responsive to the β2-antagonists ICI 118,551. The β-1/ 2- agonist isoproterenol significantly increased DNA synthesis in only ER (-) cell lines. The reduction in DNA synthesis caused by the 5-LOX inhibitor MK-886 and non-specific LOX inhibitor NDGA was significantly higher in ER (-) cell lines than in ER (+) cell lines. The COX 1/2 inhibitor aspirin and the specific COX-2 inhibitor meloxicam reduced DNA synthesis in both cell lines. The potassium (K^+) channel blocker quinidine inhibited DNA synthesis in both cell lines, and the ER (-) cell line MDA-MB-453 was more sensitive to this effect. RT-PCR experiments showed that the ER (-) cell line MDA-MB-453 expressed mRNA for the GIRK1 channel. In summary, our study suggests a link among beta-adrenergic receptor, potassium channel and AA cascade.

The observed ability of COX inhibitors, 5-LOX inhibitors, β-adrenergic blockers and K^+ channel blockers to suppress the growth and proliferation of the a subset of human breast cancer cells lines is an important finding that provides the basis for the exploration of such agents in the clinical management and prevention of breast cancer. Because these inhibitors are already widely used for the treatment of various diseases they can immediately be used in clinical trials with breast cancer patients. In particular the β-ARs represent a promising new target for the development of new drug candidates with potential application in the clinical fields because a wealth of information is already available on the biochemical and molecular events underlying signaling by these receptors.

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

CHAPTER PAGE

LIST OF FIGURES

LIST OF FIGURES

FIGURE PAGE

LIST OF FIGURES

LIST OF TABLES

TABLE PAGE

LIST OF ABBREVIATIONS

AA: Arachidonic acid

AC: Adenylyl cyclase

AF-1: Activating factor-1

AR: Amphiregulin

β_2-AR: Beta-2-adrenergic receptor

BCA: Bicinchronic acid

BRCA: Breast cancer associated gene

c-AMP: cyclic-adenosine monophosphate

CO_2 : Carbon dioxide

COX: Cyclooxygenase gene

CPM: Count per minute

CRE: c-AMP response element

CREB: c-AMP response element binding protein

CREM: c-AMP response element modulator

DAG: Diacylglycerol

DMEM: Dulbecco's modified eagle's media

DMSO: Dimethylsulfoxide

DNA: Deoxyribonucleic acid

E: Estradiol, estrogen

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

ER: Estrogen receptor

ERE: Estrogen response element

ERK: Extracellular regulated kinase

FBS: Fetal bovine serum

FGF: Fibroblast growth factor

FLAP : Five lipoxygenase activating protein

GIRK: G-protein inwardly rectified potassium channel

GPCR: G-protein coupled receptor

Grb2: adaptor protein

HER-2: human epidermal growth factor receptor 2

HGF: Hepatocyte growth factor

HRT: Hormone replacement therapy

IGF-1/2: insulin growth factor-1/2

IP_3: Inositol 1,4,5, triphosphate

LA: Linoleic acid

LOX: Lipoxygenase gene

LPA: Lysophosphatidic acid

LPS: Lipopolysaccaride

LT: Lekotrienes

MAPK: Mitogen activated protein kinase

MDGF-1: Mammary derived growth factor-1

MEK: MAPK/ERK kinase

MEKK: Mitogen/extracelllar-signal-regulated kinase kinase kinase

mER: Membrane estrogen receptor

Myc: Nuclear phosphoprotein

c-myc: Nuclear phosphoprotein gene (transcription factor)

NF-IL-6: Nuclear factor–interleukin-6

NFκB: Nuclear factor kappa-B

NNK: 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone

NSAID: Non-steroidal anti-inflammatory drugs

PAGE: Polyacrylamide gel

PBS: Phosphate buffered saline

PDGF- A/B: Platelet derived growth factor-A/B

PCR: Polymerase chain reaction

PG: Prostaglandin

PKA: Protein kinase –A

PKC: Protein kinase –C

PLA: Phospoholipase-A

PLC: Phospholipase-C

PMSF: Phenylmethylsulfonyl fluoride

PR: Progesterone

PUFA: Poly-unsaturated fatty acid

Raf-1: Cytoplasmic serine / threonine protein kinase

Ras: Membrane associated GTP protein kinase

RGD : Arginine-glycine-aspartate

RIPA: Cell lysing buffer

RTK: Receptor tyrosine kinase

RT-PCR: Reverse transcribed-polymerase chain reaction

SDS: Sodium dodecyl sulfate

Ser: Serine, amino acid

TBS: Tris buffered saline

Thr: Threonine, amino acid

TGFα: transforming growth factor alpha

Tyr: Tyrosine, amino acid

VEGF: Vascular endothelial growth factor

CHAPTER 1: INTRODUCTION

Modulators of Breast Cancer Risk:

Breast cancer is the leading type of cancer in women and ranks second after lung cancer as the cause of cancer death. Estimates from the American Cancer Society indicate that about 192,200 new cases of invasive breast cancer will be diagnosed among women in the United States and more than 40,000 deaths will be attributed to this disease in 2001. In the State of Tennessee, about 4,200 new breast cancer cases will be diagnosed in females and, the estimated number of deaths from breast cancer will be about 900 in 2001 (1,2).

A large number of risk factors for breast cancer have been identified (Table 1), even though some of these reports have yielded contradictory results (1-11). Of immediate relevance to this project are the findings that smoking and drinking as well as caffeine are risk factors for breast cancer whereas the long-term treatment of arthritis with non-steroidal anti-inflammatory agents (NSAIDS) has protective effects. Tobacco smoke contains the carcinogenic nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), which is a high affinity beta-adrenergic agonist while nicotine stimulates the release of the physiologic beta-adrenergic agonists epinephrine and norepinephrine from the adrenal medulla (12-14). Studies in human adenocarcinomas of lungs and pancreas have shown that beta-adrenergic stimulation by NNK or classic beta-adrenergic agonists increases DNA synthesis and arachidonic acid (AA) release (15). If similar pathways are expressed in adenocarcinomas of the breast, the chronic

Table 1: The important risk factors identified in breast cancer:

1. Female Gender

2. Higher incidence in Western nations

3. Black Women

4. Previous breast cancers or carcinoma in situ or atypical hyperplasia

5. Family History

6. Hereditary Breast Cancer
   - BRCA1 (Breast cancer associated gene 1)
   - BRCA2 (Breast cancer associated gene 2)
   - BRCA3 (Breast cancer associated gene 3)
   - P53
   - Ataxia-telangiectasia

7.Estrogens (Contradictory Data)
   - The longer a woman is exposed to estrogen the higher is the risk of breast cancer
   - Early Menstruation (Before age 12)
   - Late Menopause (After age 55)
   - Having no children or having them after age of 30
   - Pregnancy
   - Miscarriage and Abortions
   - Subsequent Births

8.HRT (Hormone Replacement Therapy)
   - Estrogen alone
   - Estrogen with Progesterone
   - Tamoxifen

Table 1: (continued) The important risk factors identified in breast cancer:

9.Chemicals that mimic estrogens
(Xenoestrogens: Dioxin, pesticides, plasticizers, and polystyrenes)

10.Hormonal contraceptives

11.Heavy Dense Bones – Estrogen helps build bone mass.

12.Chemical carcinogens: Polycyclic Aromatic Hydrocarbons

13.Radiation

14.Alcohol consumption

15.Reduced Melatonin-Higher breast cancer incidence in flight attendant

16.Postmenopausal obesity

17. A diet high in fat:
a) Linoleic Acid (ω-6 –polyunsaturated fatty acids i.e., soy, corn, sunflower and other vegetable oil)—BAD FAT.
b) Linolenic acid (ω-3-monounsaturated fatty acids i.e., fish, olive, canola oils)-GOOD FAT.

stimulation by beta- adrenergic agonists contained in tobacco may contribute significantly to the increased risk for breast cancer observed in smokers. Moreover, ethanol and caffeine both increase intracellular levels of cyclic-adenosine mono-phosphate (c-AMP), an effect that enhances beta-adrenergic signaling (16-18). Beta-adrenergic stimulation causes the release of arachidonic acid in human mammary, human pulmonary and pancreatic adenocarcinoma cell lines, resulting increased DNA synthesis and cell number that were significantly reduced by beta-blockers or NSAIDs that block AA metabolism by cyclooxygenase (COX) enzyme (15,19-29). AA is derived from the n-6 polyunsaturated fatty acid (n-6 PUFA) linoleic acid (LA). High levels of n-6 PUFA in the diet have been associated with an increased risk for the development of breast cancer and promote the development of breast cancer in animal models via increased synthesis of eicosanoids. By contrast, n-3 PUFAs have suppressive effects on the development of breast cancer (30,31). Unlike 2-series prostaglandins (PGs) and 4-series leukotrienes (LTs) produced by the tumor promoter n-6 PUFAs, the n-3 PUFAs (alpha linolenic acid) produces 3-series PGs and 5-series LTs that do not promote cancer formation (32,33). Linoleic acid (LA) stimulated the growth of two ER (-) breast cancer cell lines, MDA-MB-231 and MDA-MB-435, in athymic nude mice and tumor progression was suppressed by the COX inhibitor indomethacin (33).

The n-3 PUFAs are found mainly in fish oil, olive oil, flaxseed oil, canola oil, and marine food while n-6 PUFAs are found in soy, safflower, sunflowers and corn oil. The high proportion of seafood consumed by Asian women is responsible for their lower risk to develop breast cancer. The n-3 PUFAs comprise α-linolenic acid, eicosapentanoic, and docosahexanoic acids and have been shown to suppress breast cancer. The n-6

PUFAs, on the other hand, consist of linoleic, and arachidonic acids and may induce breast cancer formation (34-36). The dietary ratio of n-6: n-3 is an important factor that determines the risk for breast cancer development (34). The presence of n-3 fatty acids decreases the eicosanoids production both through competition with AA as a substrate for COX-2 and through the inhibition of the conversion of LA to AA (32).

Collectively, these epidemiological and experimental data provide the rational for the current project, which focuses on the role of beta-adrenergic, AA-mediated growth regulation of human breast cancer cell lines.

Growth-Regulating Pathways Expressed in Breast Epithelia and Breast Cancer:

Estrogen (E) plays an important role in human breast development and in the normal physiological functioning of the adult breast tissue because it is required for normal ductal development and induction of progesterone (PR) expression (1,37-39) (Figure 1). Expression levels of estrogen receptors (ERs) are high in some breast cancers while being low in normal breast epithelium adjacent to the malignant lesions (40,41). The connection between estrogen and breast cancer has been recognized for more than 100 years (42). The cumulative lifetime exposure to endogenous (and in a small degree to exogenous) estrogen is one of the important determinants of breast cancer risk. In animal model systems, estrogen induced and promoted mammary tumors, and removing animals' ovaries suppresses the effect of estrogen (37). Two clinically different types of breast cancer are recognized: breast cancers that express the ER (ER (+) or estrogen-responsive) and breast cancers that do not express the ER (ER (-).

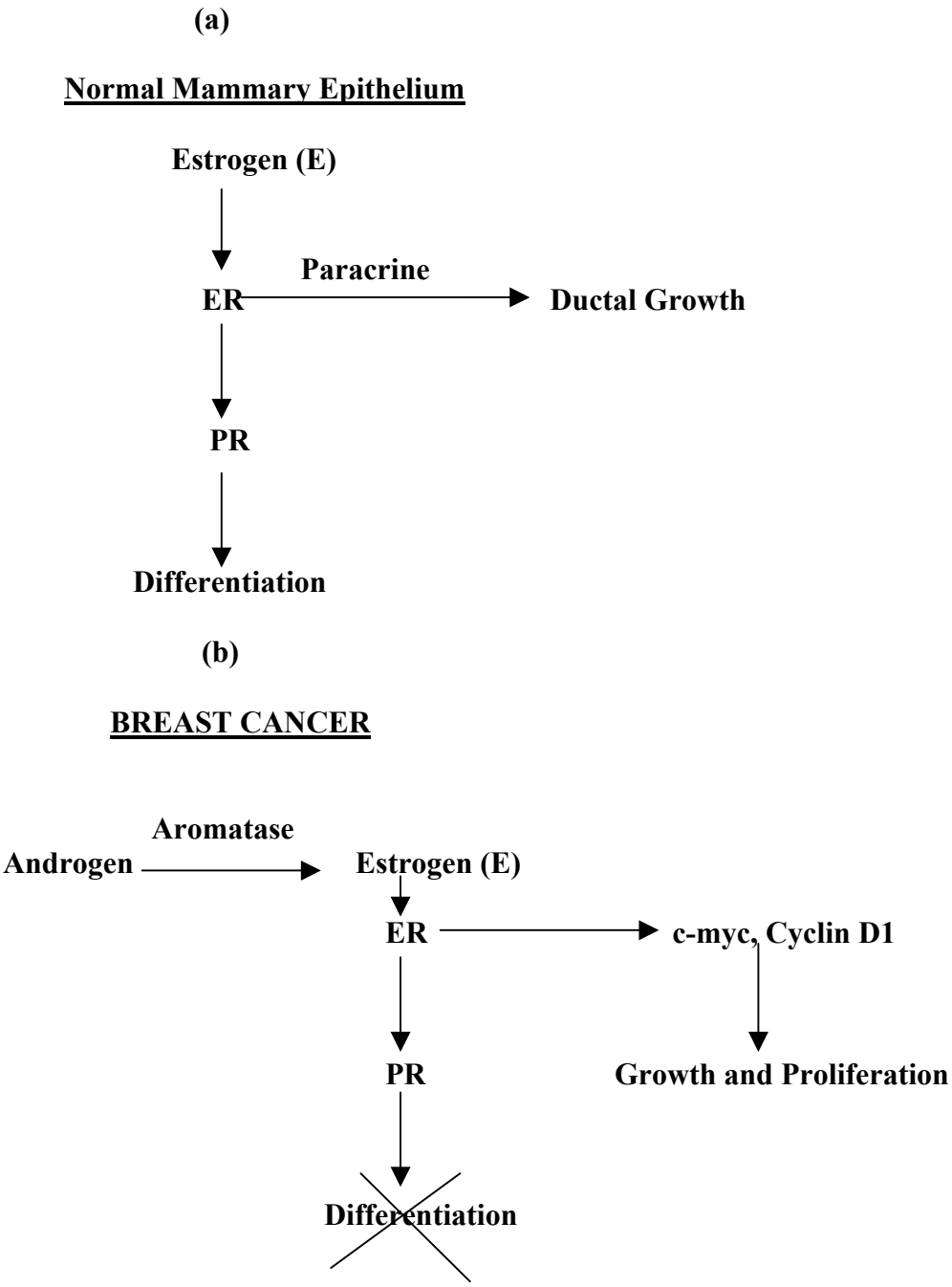


Figure 1: The role of estrogen and the ER on breast development and cancer. In normal breast tissue, estrogen induces ductal growth via a paracrine loop and progesterone (PR) expression so that mammary differentiation may occur (a). However, during breast cancer development, estrogen blocks differentiation and induces c-myc and cyclin D1 genes to stimulate growth and proliferation (b).

Immunohistochemical studies have shown that about 70 % of breast cancers stain positively for ER (43). These tumors are often more differentiated, less invasive and respond to anti-estrogen therapy. On the other hand, nearly 30 % of breast cancers have no ER as demonstrated by immunohistochemical and radio-ligand binding assay. These tumors tend to be more aggressive and rarely respond to hormonal therapies since they may grow in the absence of estrogen (44).

The mechanism how estrogen contributes to the development of breast cancer is poorly understood. Alkylation of cellular molecules and the generation of active radicals that damage DNA by estrogen and/or its metabolites may be involved (42,45-47). Estrogen has direct and indirect proliferative effects as demonstrated in studies using human breast cancer cell lines. Direct action may occur through the activation of oncogenes, inactivation of tumor suppressor genes and induction of genes or proteins involved in DNA synthesis, whereas indirect action may occur through the interaction with peptide and non-peptide growth factors (42).

In addition to the nuclear ER through which estrogen exerts its effect on DNA, a membrane estrogen receptor (mER) has been recently identified through which estrogen may interact with tyrosine kinase receptors in the ras / raf / MAPK (Mitogen activated protein kinase) signaling pathway (48-50). This mER operates as a G-protein coupled receptor (GPCR) that has been shown to mediate Ca^{+2} mobilization and Ca^{+2} influx in mouse macrophages in vitro (51). Consistent with this, E binds to mER to activate a variety of signaling cascades: a) stimulation of the AC-c-AMP-PKA (protein kinase –A) pathway by inducing G_S subunits of G-proteins, b) activation of PLC (phospholipase-C) resulting in increases in intracellular calcium (Ca^{+2}) concentration and protein kinase-C

(PKC) through the stimulation of G_q subunits of G-proteins, c) activation of the MAPK/ERK (extracellular regulated kinase) pathway through Src or Ras, d) activation of *fos* expression through ERK (52).

Locally acting polypeptide hormones or growth factors modulate the regulation of breast tissue by estrogen and progesterone. These growth factors regulate cell growth by activating intracellular signaling pathways after binding to high affinity tyrosine kinase receptors on the cell surface (Figure: 2). Among these, the MAPK pathway plays an important role in the transduction of mitogenic signals initiated by growth factors acting on a variety of cell surface receptors. MAP kinases are regulated through many protein phosphorylation cascades.

Several stimulatory growth factors are found in breast cancers including, epidermal growth factor (EGF), transforming growth factor alpha (TGFα), insulin growth factor-1/2 (IGF- 1/2), platelet derived growth factor-A/B (PDGF- A/B), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor, (HGF) and mammary derived growth factor-1 (MDGF-1) (53-57). The EGF receptor (EGFR) is a 170 kDa transmembrane receptor with tyrosine kinase activity, which has several ligands including EGF, amphiregulin (AR) and TGFα (53). EGF and the EGFR are frequently over-expressed in ER (-) breast cancers. When the ER (+) human breast cancer cell line MCF-7 was transfected with an anti-sense estrogen receptor RNA, the ER levels decreased whereas EGFR expression increased, suggesting that loss of expression of ER may render breast cancer cells to have concomitant EGFR increases or activate EGFR expression by inducing cellular cascades (58).

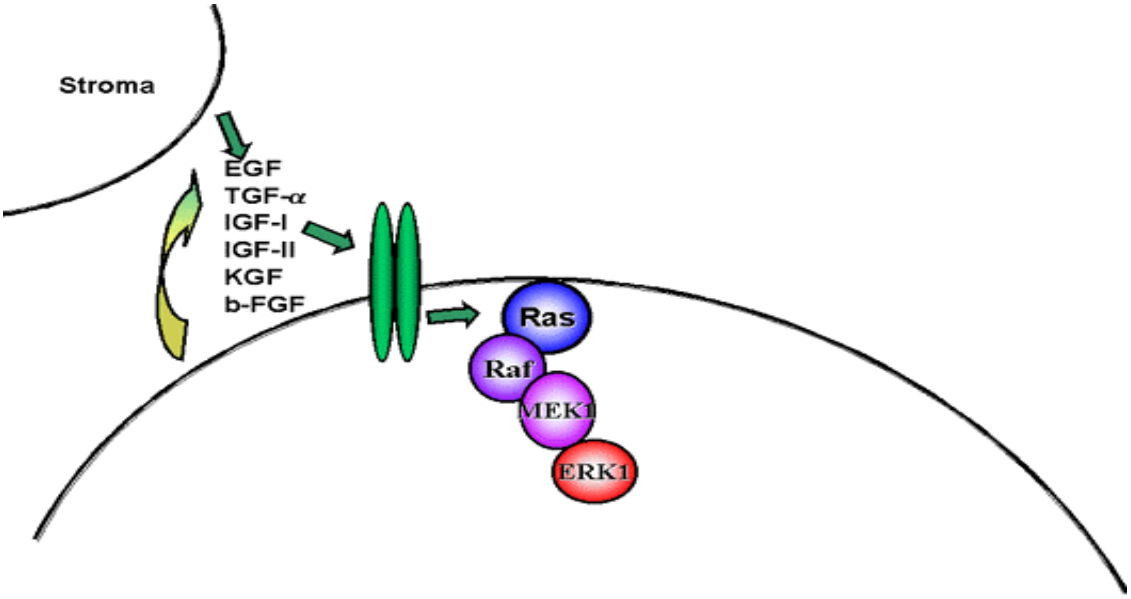


Figure 2: Several peptide growth factors activate the MAPK pathway in ER(-) breast cancer cells. The MAPK pathway can also be stimulated by mutant Ras and by autocrine and paracrine growth factor loops implicated in cancer growth.

Agents inducing c-AMP stimulation (okadaik acid, forskolin) stimulate ligand-independent transcriptional activation of the ER and synergize with the ER-mediated activation (59). Cross talk between the ER and PKA pathway has been observed and stimulation of PKA activates the agonist activity of antiestrogens in ER (+) breast cancers (59-62). Up-regulation of PKA activity leads to ligand-independent activation of ER and increased phosphorylation at the activating factor-1 (AF-1) site of the ER gene (63-65).

On the other hand, prolonged exposure of the ER (+) human breast cancer cell line MCF-7 to high c-AMP levels led to a reduction in the number of estrogen receptors (66). These findings suggest that PKA or c-AMP activators and inhibitors may modulate the development of breast cancer because estrogen may act through the c-AMP system to regulate c-AMP-mediated gene expression. This may have potential importance in the beta-adrenergic regulation of breast cancers investigated in this project, as these receptors often activate cAMP/PKA signaling pathway.

The arachidonic acid (AA)-metabolizing enzymes cyclooxygenase-2 (COX-2) and lipoxygenases (LOXs) are frequently over-expressed in ER (-) breast cancers, suggesting a role of the AA-cascade in the development of this malignancy (22,23,27,67-70). Specific AA metabolites have been reported to modulate the proliferation and development of several cancers, including breast cancer (Figure: 3). Activation of phospholipase-A_2 ($cPLA_2$) represents the rate- limiting step in the production of different eicosanoids because the resting level of AA is normally low within the cells (71,72). The cPLA2 translocates from the cytosol to the endoplasmic reticulum and perinuclear membranes where COXs and LOXs are localized (72,73). PLA2 isoforms are over-expressed in pancreatic and lung adenocarcinoma cells (74,75). Phosphorylation of

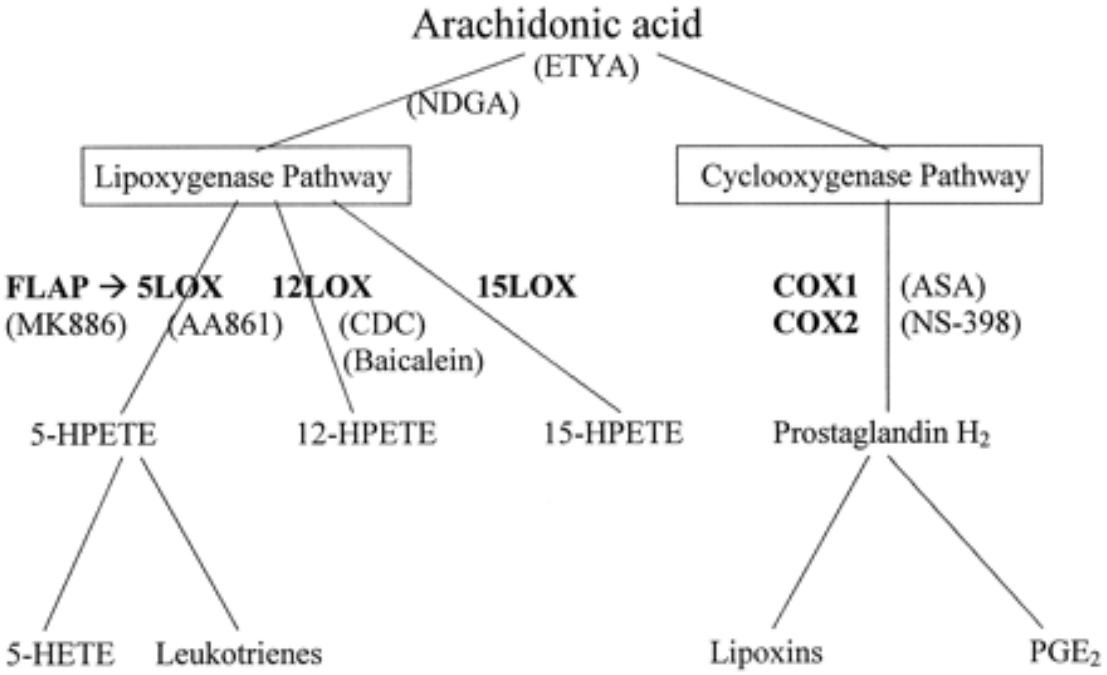


Figure 3: The Arachidonic acid (AA) cascade and several chemicals (in brackets) inhibiting the formation of AA metabolites.

cPLA2 by oncogenic Ras and MAP kinase ERK2 in cells over-expressing cPLA2 and ERK2 may cause the release of AA from membrane phospholipids (71,76). AA induced MAPK pathway in the ER (-) human breast cancer cell line, MDA-MB-435 (21). The interaction of EGF with its receptors in breast cancer cells can also lead to the hydrolysis of phospholipids and release of fatty acids, such as AA. Several LOX products not only have mitogenic effect but they also activate several oncogenes (77-79).

The induction of AA metabolism is a characteristic response to tissue damage and irritation and may contribute to epithelial tumor promotion (22,23). AA has been indicated as a potent second messenger responsible for a number of cellular events. It activates PKC, MAPK, and PKA; synthesizes heat shock proteins; activates sphingomyelinases leading to apoptosis; induces immunoglobulin G (IgG)-mediated phagocytosis; and activates NAPDH oxidase resulting in the production of the superoxide anion (80). AA has been shown to increase cellular c-AMP levels in primary epithelial cell cultures taken from C3H mouse mammary tumors (29). AA is also subject to non-enzymatic, free radical oxidation to bioactive isoprostanes and isoleukotrienes (81).

Fatty acids may also be second messenger molecules for the modulation of ion channels. This might involve direct interaction of AA with the ion channels or alteration of ion channel interactions with the plasma membrane. AA and PGs have been shown to modulate ion channels, including Ca^{+2}, K^{+1}, Cl^{-1} and Na^{+1} channels. For example, AA caused the release of Ca^{+2} from intracellular stores, activated Ca^{+2} influx from the membrane channels and activated potassium channels in smooth and heart muscle cells (82,83).

NSAIDs inhibit both COX isoenzymes, and regularly taking NSAIDs provides a 40-50% decrease in the risk of death by colon cancer (24). A link between the effects NSAIDs and COX-2 protein amplification has been suggested in some studies in that ibuprofen not only suppressed the proliferation of mammary tumors but also inhibited both the production of PGs and the expression of COX-2 protein in mammary tumors (84,85).

There are two isoforms of COX: COX-1 (a constitutive form) and COX-2 (an inducible form). COX-1 is constitutively expressed and required to maintain homeostasis. COX-2 is inducible and is over-expressed during numerous pathological conditions, such as cancer and (24,86). A large number of growth factors, cytokines, hormones and tumor promoters have been shown to induce COX-2. COX-2 is over-expressed in several cancers such as colorectal, gastric, breast, pancreatic, lung, and skin cancer (19,20,24,86-90). High levels of COX-1 have been reported in the ER-dependent well differentiated MCF-7 breast cancer cell line while ER-independent, invasive MDA-MB-231 cells showed a low expression of COX-1 but a high level of COX-2 (91,92). These findings suggest that COX-2 contributes to the growth and proliferation of ER (-) breast cancer cells that and COX-2 inhibitors may be used to control breast tumorigenesis. Another study demonstrated the over-expression of COX-2 protein in transformed mammary epithelial cells (89).

Parret et al demonstrated for the first time that COX-2 was over-expressed in primary breast cancers, not in the normal breast tissue (93). Showing that ER (-) breast cancers are highly invasive and metastatic, Liu et al suggested that the high level of both COX-2 protein and PGs found in the metastatic ER (-) breast tumors (MDA-MB-231)

might be responsible for cancer metastasis, and selective COX-2 inhibitors might be useful treatment options for this kind of breast tumors (91). This is in contrast to the very low level of COX-2 protein found in the ER (+) breast cancer cell line (MCF-7). Another study demonstrated the over-expresion of COX-2 protein in transformed mammary epithelial cells (89).

The cyclic-AMP response element (CRE) and nuclear factor kappa-B ($NF_{\kappa}B$) consensus sites on the COX-2 promoter are important for the expression of the COX-2 gene, because CRE acts as a positive regulatory element for COX-2 transcription (191). $NF_{\kappa}B$ regulates COX-2 expression in several cells and the inhibition of $NF_{\kappa}B$ results in the reduced expression of COX-2 and tumor cell proliferation in gastric cancer cells (26). In rat mammary tumors $NF_{\kappa}B$ is abnormally activated and the inhibition of constitutive $NF_{\kappa}B$ caused apoptosis (94). Previous studies have shown the importance of CRE in regulating COX-2 expression in epithelial cells. CRE mediates the effect of *src* on COX-2 expression in fibroblasts (95). In endotoxin-treated macrophages, the induction of a murine COX-2 reporter gene by lipopolysaccaride (LPS) needed a CRE site and a nuclear factor–interleukin-6 (NF-IL-6) (96). Because the promoter region of the COX-2 gene has CRE, the activation of c-AMP by β_2-adrenergic receptor (β_2-AR) agonists will likely induce the expression of the COX-2 gene whereas β_2-AR antagonists will reduce or inhibit the expression of the COX-2 gene (97). A highly specific β_2-AR agonist, zinterol, triggered AA release via the activation of the c-PLA2 in ventricular cardiomyocytes and these responses to β_2-AR induction were mediated by AA under c-AMP control (98). Inoue et al. (99) demonstrated that CRE is necessary for effective transcription of the human COX-2 gene since the deletion of this regulatory site on the

COX-2 promoter decreased the promoter activity of COX-2 gene by 80 %. This suggests that modulators of the c-AMP-PKA pathway are important in the regulation of the COX-2 gene. On the other hand, the deletion of the $NF_{\kappa}B$ regulatory site reduced the promoter activity of the COX-2 gene only by 30 % (99). In mouse osteoblastic cells c-AMP induced COX-2 expression (100), suggesting that the expression of COX-2 protein might be modulated by c-AMP either directly through PGE2, one of the end products of COX-2-mediated AA metabolism, or indirectly through the PKA pathway (88,101).

Beta-adrenergic receptors (β_2-AR) are members of the superfamily of GTP-binding protein (G protein)-coupled receptors (GPCRs; Figure: 4). GPCRs, which do not have intrinsic catalytic activity, consist of an extracellular N-terminal domain; seven membrane-spanning domains connected by three extracellular and three intracellular loops; and an intracellular C-terminal cytoplasmic tail (102-104). These transmembrane receptors regulate a host of diverse physiological processes and cellular functions, including cardiovascular function, cellular metabolism, neurotransmission, secretion, cellular growth and differentiation, ion channel activity, immune responses and proliferation of certain tumor cells (105-107). Upon binding of a ligand to GPCRs, the bound GDP exchanges for GTP on the G-proteins, leading to the dissociation of G-protein into active G_{α}-GTP and $G_{\beta\gamma}$ subunits (Figure: 4).

Three different types of beta-adrenergic receptors (β_1, β_2, β_3) have been identified (105-108). Of these, β_1- and β_2-receptors are expressed in breast epithelia while β_3-receptors are expressed in adipose tissue. While all three beta-adrenergic receptors may activate adenylyl cyclase/cAMP (Figure 5,6), the β_2-receptor may additionally activate

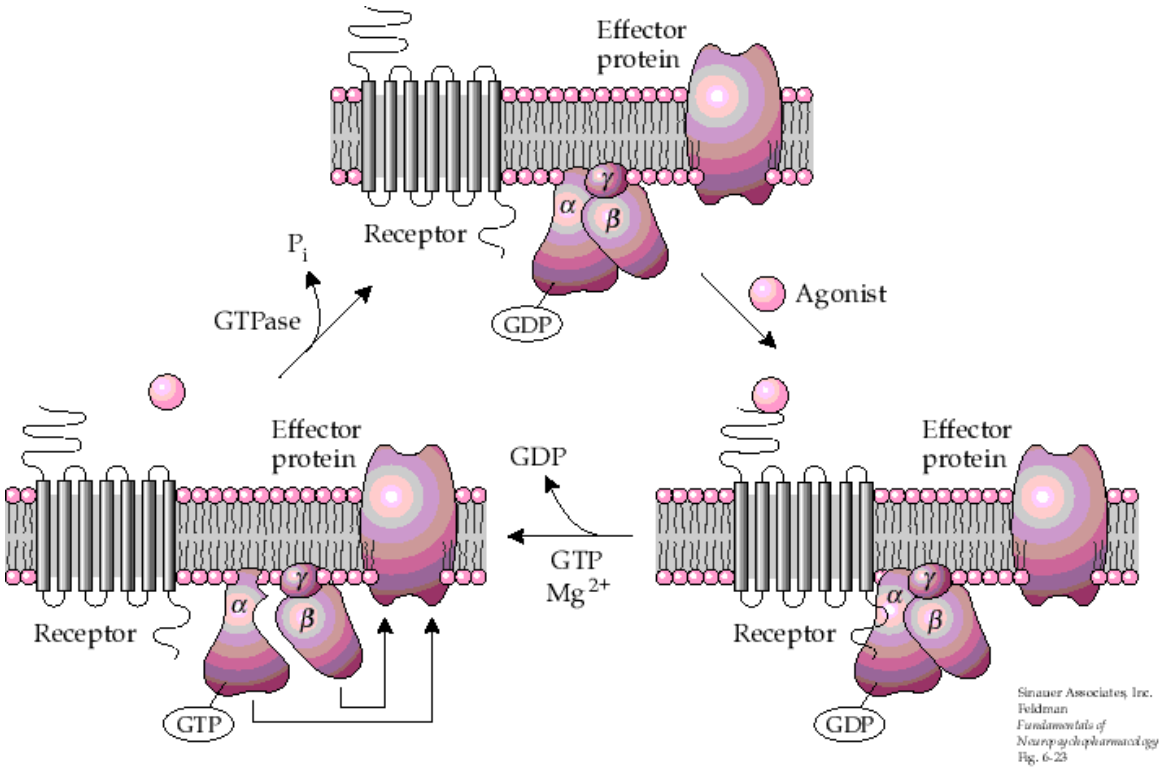


Figure 4: Mechanism of action of G proteins and GPCR. When an agonist binds to GPCRs, the bound GDP exchanges for GTP on the G-proteins, leading to the dissociation of G-protein into active G_{α}-GTP and $G_{\beta\gamma}$ subunits. Once the agonist leaves the receptor, the subunits associate each other again to form inactive GDP bound state.

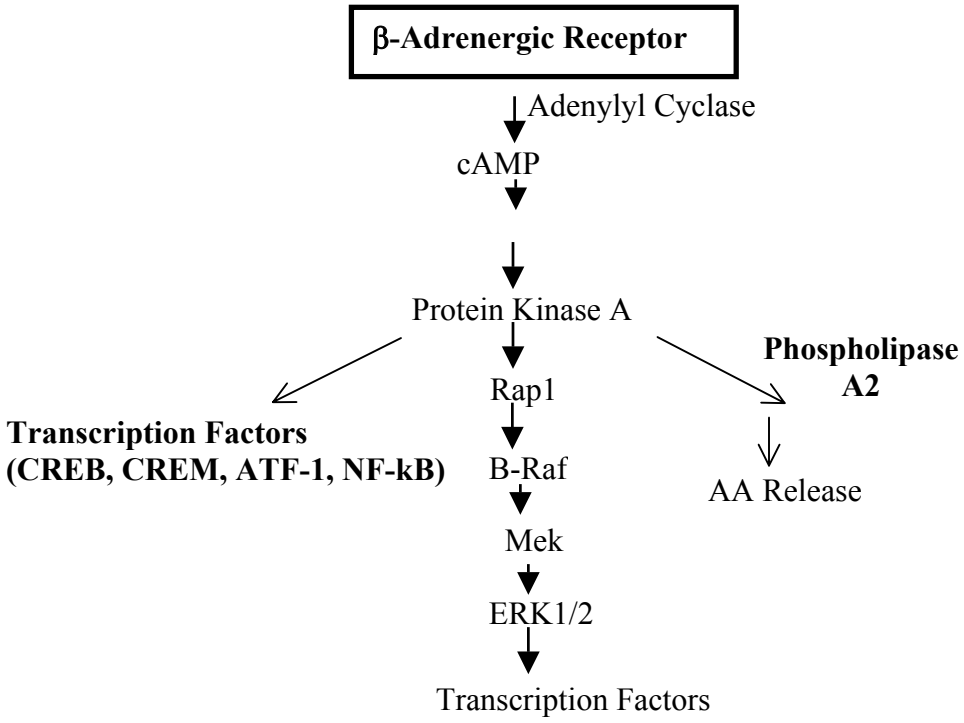


Figure 5: cAMP-dependent signal transduction in response to β-adrenergic receptor stimulation. Binding of agonists to the receptor activates the enzyme adenylyl cyclase, resulting in activation of the second messenger c-AMP and its associated kinase protein kinase A (PKA). In turn, PKA may phosphorylate the transcription factors CREB, CREM, ATF-1, or NF-kB or the MAPK cascade via activation of Rap-1 and b-Raf or it may cause the release of AA via activation of phospholipase A2.

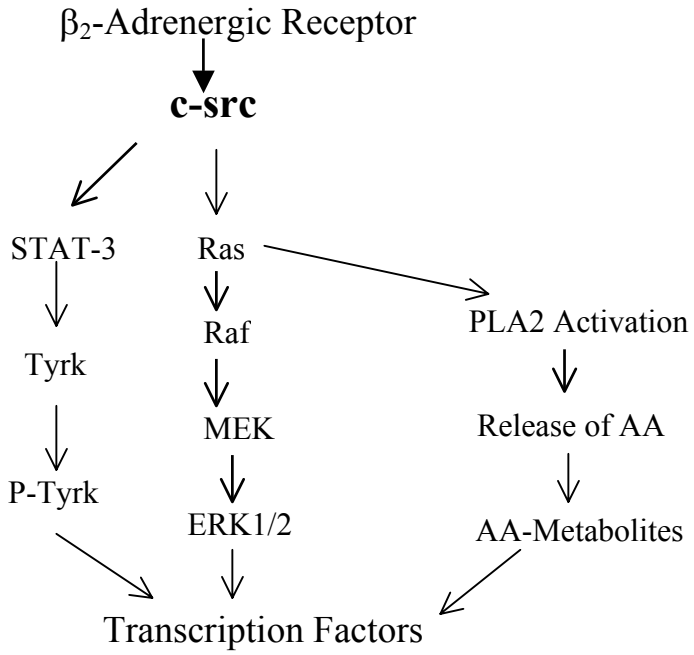


Figure 6. Beta$_2$-adrenergic signal transduction via c-src. Pending on the cell type, binding of agonists to β_2-adrenergic receptors may recruit and activate c-src. Activation of c-src may result in induction of the mitogen-activated protein kinase (MAPK) pathway via *ras* to cytoplasmic serine/threonine kinases Raf, Mek, ERK and RSK, leading to induction of transcription factors such as AP-1 and c-myc (101, 116). Additionally, ras may cause the release of AA via activation of phospholipase A. Alternatively, induction of c-src activity may results in tyrosine -phosphorylation and activation of Stat 3 (signal transducer and activator of transcription 3).Tyrosine phosphorylation of STAT 3 facilitates its dimerization and translocation to the nucleus, leading to the transcription of its target genes.

the c-src/Stat-3 pathway (Figure 6) or potassium channels (109-115). Activation of PKA as well as c-src can induce the release of AA via activation of phospholipase A_2 (22,41,72,76) proliferation stimulated by NNK (15). Moreover, the AA release induced by NNK was completely inhibited by the β_1-AR antagonist atenolol and β_2-AR antagonist ICI 118,551 (15). This data suggests that the potential up-regulation of β-ARs together with AA release may be the sole contributing factor for the lung tumorigenesis and β-AR-AA-dependent regulatory pathways might be considered as a crucial target for cancer prevention studies.

Similar to other G protein-coupled receptors, β-adrenergic receptors may also activate G-protein-activated K^{+1} Channels (GIRK or $K_{ir}3$) by binding to a membrane-bound effector complex, which includes α and β subunits of G proteins in addition to the effector channel. The α and β subunits possess some effector specificity and activate the channel directly (117-120). Activation of heterotrimeric G-proteins by their coupled receptor leads to dissociation of the G protein α and βγ subunits. Direct interactions of G_α or $G_{\beta\gamma}$ with effector proteins transduce the external signal into an intracellular response (Figure: 4).

The G-protein-activated K^{+1} channels (GIRK or $K_{ir}3$) are one of six groups comprising the family of inward rectifying K^{+1} channels. As indicated by their name, GIRKs are activated by G proteins, most of which modulate many ion channels through second messenger cascades (Figure 7). GIRKs are activated via direct interaction of the channel with $G_{\beta\gamma}$ subunits released from β-adrenergic receptors and $G_{\beta\gamma}$ subunits

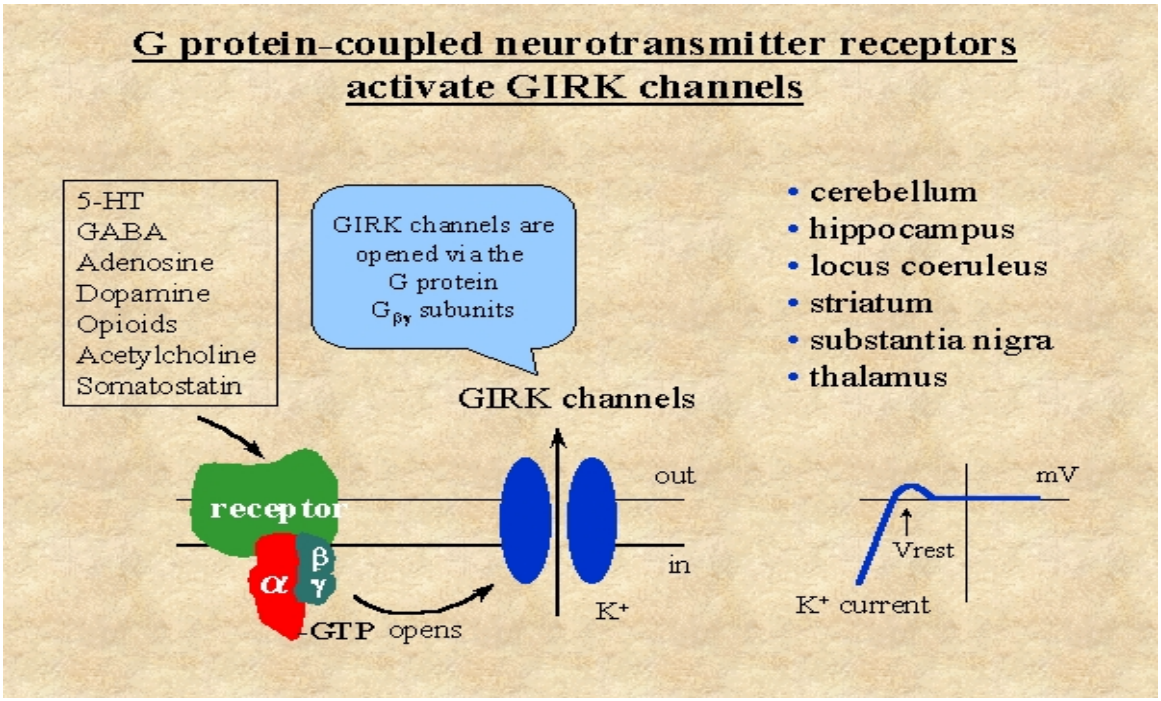


Figure 7: GIRK channel activation. GIRKs are activated by G proteins most of which modulate many ion channels through second messenger cascades.

induce both native and recombinant GIRK channels by binding directly to the N- and C-terminal regions of GIRK1. Intracellular Na^{+} and Mg^{+2} ions also activate these channels. (117-120).

In Xenopus oocytes, where the GIRK1, $G\alpha_S$ and β_2-AR were co-expressed, isoproterenol activated GIRK1 channel activation and coupling between GIRK1 and β_2-AR, which was independent from the PKA pathway, but through the release of G protein $\beta\gamma$ subunits, suggesting that $G_{\beta\gamma}$ subunits released from β-ARs activate the basal GIRK current (118). However, β_2-AR activation by isoproterenol elevated the acetylcholine-induced GIRK current through β_2-AR, $G\alpha_S$ and PKA- mediated phosphorylation in atrial cardiomyocytes, indicating that PKA phosphorylation of β_2-AR might permit GIRK channels to open either by direct phosphorylation of GIRK or activation of the auxiliary modulators interacting with the GIRK channel (121). The stimulatory effect of PKA phosphorylation on the GIRK might partly be explained by a PKA phosphorylation consensus site found in the carboxy end of GIRK1 (119). The exact physiological role of this site, which may be responsible for activation of several GIRK channels by growth factors or kinases, remains to be elicited in the future.

Various effects of AA products called as eicosanoids and fatty acids have been demonstrated on GIRK channels (82,122-129). Induction of the endothelin receptor inhibited GIRK channels via an AA and PLA2 mediated mechanism in Xenopus leavis oocytes (122). Several free fatty acids including AA, oleic and linoleic acid inhibited ATP-dependent gating of G-protein –gated K^{+1} channels and GIRK1/4 recombinant channels in oocytes (123). AA and eicosanoids have direct effects on the GIRK

channels by modification of the channel structure (125). It seems that the mechanism by which free fatty acids are modulating many ion channels including GIRK may partly be explained by high concentrations of total free fatty acids near the inner surface of the membrane near the channels, as free fatty acids diffuse through cell membranes partly in the unbound form (129).

Recently, over-expression of GIRK1 has been reported in a large proportion of malignant breast cancers, particularly ER (-) cancers. Because GIRK is involved in signal transduction initiated by β_2-adrenergic receptors in some cell types (130), its over-expression in breast cancer tissues suggests a potential role in beta-adrenergic growth regulation of breast cancer cells.

In summary, several pieces of evidence suggest that beta-adrenergic receptors, c-AMP, GIRK channels, and the AA-cascade may all work in concert to regulate the growth of breast cancer cells and that components of these pathways are selectively stimulated by several of the key risk factors that have been identified for the disease.

Hypothesis:

The central hypothesis of this research dissertation is that beta-adrenergic receptors stimulate the growth of human breast cancer cell lines via activation of the arachidonic cascade and GIRK channels. If this hypothesis is true, beta-blockers will inhibit cell proliferation, MAPK and GIRK expressions, whereas beta-agonists will induce cell proliferation through beta- adrenergic receptors. COX-1/2 and 5-LOX inhibitors will block cell proliferation through the arachidonic acid cascade while potassium channel blockers will inhibit cell proliferation via GRK channels.

Specific aim:

Determine the effects of different concentrations of beta-blockers, beta-agonists, inhibitors of COX-2, 5-LOX, and potassium channels on cell proliferation, MAPK and GIRK expressions in a panel of ER (+) and ER (-) human breast cancer cell lines.

CHAPTER 2: MATERIALS AND METHODS

Cells:

ER (+) human breast cancer cell lines MCF-7, ZR-75-1, MDA-MB-361, and ER (-) human breast cancer cell lines MDA-MB-453, MDA-MB-435, MDA-MB-231, MDA-MB-468 were obtained from the American Type Culture Collection. All cells were grown to 70-80% confluency in 75 cm^2 vented cell culture flasks before experimental procedures. All cells were maintained in RPMI 1640 media with 10 % FBS supplemented with L-glutamine (2mM (210 mg)) (Life Tech.) and penicillin (100U/ml) / streptomycin (100 μg/ml) (Life Tech.) in an atmosphere of 5 % CO_2 at 37°C.

Chemicals:

The site-selective antagonists for β_1-and β_2-adrenergic receptors atenolol and ICI 118,551 were purchased from Tocris. The broad-spectrum β-adrenergic antagonist propranolol and the COX inhibitor aspirin were purchased from Sigma. The β-adrenergic agonist isoproterenol was purchased from RBI. The five lipoxygenase activating protein (FLAP) inhibitor MK-886 was purchased from Biomol Research Lab., PA. The non-specific LOX inhibitor NDGA, COX-2 inhibitor meloxicam and K-channel inhibitor quinidine were purchased from Calbiochem. Tritiated thymidine was purchased from Amersham.

[^{3}H] –Thymidine Incorporation Assay:

DNA synthesis was assessed with all cell lines using a standard tritiated thymidine incorporation procedure in 96 well plates with a final volume of 200 µl/well with a tritiated thymidine concentration of 0.5 µl / well (specific activity: 0.5 µCi / 200ml). Initially, cells grown to 80% confluency were trypsinized and 15 000 cells were plated into 96 well plates with DMEM medium including 10% FBS, 210 mg L-glutamine, 500 units penicillin, and 500 µg streptomycin was used for the inhibition studies and low serum (0.1 % FBS) DMEM medium was used for the stimulation studies. Cells were put into the incubator for 4 hours prior to the treatments. Cells were treated with increasing concentrations of propranolol, atenolol, ICI 118,551, isoprotorenol (50nM, 100nM, 250nM, 500nM, 750nM and 1µM), MK-886 (100nM, 1µM and 10µM), aspirin (1µM, 10µM and 100µM), NDGA (100nM, 500nM, 1µM, 5µM, 10µM, 20µM, 30µM, 40µM), meloxicam (1µM, 10µM, 20µM, 30µM, 40µM, 60µM, 80µM, 100µM, 150µM, 200µM), and quinidine (10µM, 20µM, 40µM, 60 µM, 80µM, 100µM, 120µM) for 24 hours at $37C^0$. Cells, then, were lysed by using 0.1N NaOH and incorporated [^{3}H]-thymidine was separated from non-incorporated thymidine by vacuum filtration using a microplate harvester (Micromate 196, Packard) of the lysed cells onto a backed glass fiber filter. DNA was adhered onto filter by a final flush with 200µl/well of isopropanol. The filters on which DNA adhered were cut by the harvester apparatus into circular discs corresponding to each well of 96 well plates. Each disc was placed into a glass liquid scintillation vial including 3 ml of counting cocktail (Bio-Safe II, Mount Prospect, IL). Radioactivity bound to the filters was determined by liquid scintillation counting.

Relative-RT-PCR:

ER (-) MDA-MB-453 breast cancer cells were placed in DMEM media including 10 % FBS in the incubator 1 hour prior to the experiment. Cells were treated with 1μM propranolol continuously for 1 week or 30 min every day for 1 week while maintained in 5 % CO_2. Cells were trypsinized, and RNA was isolated by Trizol reagent (Invitrogen-Life Technologies, Grand Island, NY). Cells were further purified to remove DNA with an Absolutely RNA kit (Stratagene, La Jolla, CA). For the RT reaction, 2 μg DNase I (Life Technologies) treated RNA, 1 μg random decamer primers (Ambion, Austin, TX) and nuclease free water were heated to 70^{o} C for 3 min., then placed on ice. To this was added 0.5 mM each dNTP, 10 mM DTT, 30 U Prime RNase inhibitor (Eppendorf, Westbury, NY), 200 U Moloney murine leukemia virus (M-MLV) reverse transcriptase (Life Technologies), PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2) and nuclease free water in a final volume of 20 μl. This was incubated at 37^{o} C for 1 hour, and then the enzymes were heat inactivated for 10 min. at 92^{o} C. A negative control reaction was also performed without the M-MLV to determine if there was genomic DNA contamination. PCR was done with 5 μl of the RT reactions. This was mixed with 0.2 mM dNTPs,, PCR buffer 1.25 U Ampli-Taq polymerase (Applied Biosystems, Foster City, CA), DMSO, primers for GIRK-1 (500 nM), 5 μCi [α-^{32}P]-dCTP (3000 Ci/mmole, Dupont-NEN, Boston, MA) and nuclease free water in a final volume of 50 μl. The GIRK-1 primers are forward 5′-ctatggctaccgatacatcacag-3′ and reverse 5′-ctgttcagtttgcatgcttcgc-3′, which span exon 1 and 2 (Schoots et al., 1997) and amplifies a 441 bp fragment (bases 631-1072, Genbank Acession # 00239).

Sequencing:

The PCR product was sequenced to verify the integrity of the PCR process. Sequencing was done with the ABI Terminator Cycle Sequencing reaction kit on an ABI 373 DNA sequencer (Perkin-Elmer, Foster City, CA). Sequences were entered into DNASIS software (Hitachi, S. San Francisco, CA). The sequence was homologous with the published sequence (131).

A preliminary experiment was done with cDNA made from MDA-MB453 cells to determine a cycle number of PCR amplification that is within the linear range, which is critical for meaningful results to compare expression levels between samples. Reactions were run on a MJ Research PTC-200 thermal cycler with the following conditions: 1 cycle of 2 min. at 94^{o} C, then samples were taken at each odd number cycle from 15-35 cycles of 94^{o} C, 30 sec; 55^{o} C, 30 sec; 72^{o} C, 45 sec. A 10 µl sample of each PCR reaction was heated at 95^{o} C for 3 min., then loaded into a 5% TBE-urea Ready Gel (Bio-Rad, Hercules, CA). This underwent electrophoresis at 200 V in TBE buffer until the xylene cyanol dye front reached the bottom of the gel. The gel was transferred to filter paper, dried and exposed to film or imaged on a Molecular Dynamics 445 SI phosphoimager (Sunnyvale, CA). For this polyacrylamide gel, the 100 bp DNA ladder was exchange labeled with T4 polynucleotide kinase and 30 µCi [γ-^{32}P] ATP (3000 Ci/mM, Dupont-NEN). A 100,000 CPM aliquot of the DNA ladder was run on the gel.

It was determined that 31 cycles of PCR amplification was in the linear range of amplification for cDNA from hamster cells. A second preliminary experiment was done with hamster cDNA. In this experiment 400 nM concentration of either 1:20, 1:15, 1:12, 1:9, 2:8 or 3:7 mixture of 18S primers/ 18S competimers (Ambion-Classic II) was added

to the above PCR mixture and the samples underwent PCR amplification for 25 cycles. The 18S ribosomal RNA primers/competimers are used as an invariant internal control which allows correction for sample variation. Electrophoresis of the PCR products was performed as described above. It was determined that a 1:9 18S primer/competimers ratio yielded a band similar in density to the gene-specific band and therefore was the proper concentration to use when mRNA expression levels of the MDA-MB453 cells were compared.

Western Blot:

For cell stimulation studies, cells were placed into low serum DMEM media (% 0.01 FBS) including 210 mg L-glutamine, 500 units penicillin, and 500 μg streptomycin for 12-24 hours and maintained in 5 % CO_2. Cells were washed with PBS and incubated for additional 30 min in low serum media in 5 %CO_2 prior to the experiment. Fresh 1 μM isoproterenol was added to the treatment group for 6 and 24 hours. For inhibition studies cell were placed in high serum DMEM media (10 % FBS) and maintained in 5 % CO_2 for 1 hour prior to the experiment. Freshly made 1 μM propranolol, atenolol and ICI 118,551 were added to the treatment groups for short and long intervals (15, 60 and 150 minutes), while 30 μM NDGA, and 100 μM meloxicam were added to the treatment groups for 24 hours. Cells were collected by removing DMEM media containing designated treatment, wash with ice-cold PBS, and adherent cells were removed of with 0.25 % trypsin. Cells were washed twice with PBS and centrifuged at 1000 rpms (60 g's) to form a cell pellet. The pellets either were placed at -80^0 C or lysed for the subsequent procedures. Cells were lysed in 600 μl RIPA solution (1 x PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) including protease inhibitors:

10 µl/ml phenylmethylsulphonyl fluoride (PMSF-10 mg/ml), 10 µl/mg sodium orthovanadate (Na_3VO_4-100mM), and 30 µl/ml aprotinin (1.9mg/ml). The cell lysate was passed three or four times through a 21 gauge needle with subsequent addition of 10 µl PMSF (10mg/ml) followed by a 30 minute incubation on ice. The cell lysates were centrifuged at 14000 rpms (1600 g's) for 20 minutes at 4^0 C to separate supernate and pellet. The supernate was collected to be used for subsequent assays. The protein in the supernate was concentrated by using Centricon^{TM} 10 concentration columns (Milipore) and centrifuged at 6000 rpms (4350 g's) for 1 hour. The protein concentration was determined by using BCA^{TM} protein concentration assay. The 10 µl protein sample and 200 µl bicinchronic acid (BCA^{TM}) reagent were placed into 96 well microplate wells and incubated for 30 minutes at 37^0 C. The protein samples were quantified to obtain absorbance values in an EL 312 microplate reader (Bio-Tek Instruments) at 550 nm wavelength. These absorbance values were converted to µg/ml by using Prism 2.01 (GraphPad Inc.) regression analysis to calculate the necessary amount of protein to load.

Protein (20 µg) for each sample was boiled in 2x SDS loading buffer (4% SDS, 0.1 M Tris-Cl at pH 6.8, 0.2% bromophenol blue, 20% glycerol) for 4 minutes at 95^0 C and samples were immediately loaded onto a 10% Tris-HCl PAGE gel (Biorad) placed in the electrophoresis apparatus (Biorad). The gel was allowed to run at 200 volts for 30-45 minutes. The gel including proteins was removed from the electrophoresis apparatus and was carefully placed in the transfer apparatus (Biorad). The gel was electrophoretically transferred to a nitrocelluse membrane (Midwest) in transfer buffer (5.8 g Tris-hydroxymethyl-aminomethane, 2.9 g glycine, 3.7 ml 10% SDS, 200 ml

ethanol, 800 ml deionized water) and run at 100 volts for 1 hour. The membranes were carefully removed from the apparatus and washed three times in wash solution (TBS including 0.05% tween-20). The membranes were blocked in blocking solution (5% nonfat dry milk in TBS/0.05% tween-20) for 1 hour at room temperature, followed by three 5 minute washes in wash solution. Following blocking and washing, the membranes were incubated for 1 hour, at room temperature, in blocking solution containing rabbit polyclonal IgG for ERK 1/2 (200 μg/ ml) (Santa Cruz Biolabs) at a dilution of 1:2000. The membranes were washed three times for 5 minutes each and incubated in blocking solution (5 % nonfat dry milk TBS/0.05% tween-20) including anti-rabbit IgG-horse radish peroxidase linked secondary antibody (400 μg/ml) (Santa Cruz Biolabs) at a dilution of 1:1000 for 1 hour at room temperature. Positive immunoreactivity was detected with the aid of western blot Luminol reagent (Santa Cruz). The time for exposures varied.

For phosphorylated ERK 1/2 antibodies (phosph-p44/42 MAPK), the membranes were blocked in a blocking buffer including 5% nonfat dry milk in TBS/0.1% tween-20 for 1 hour at room temperature. Following three separate 5 minute washes in TBS/0.1% tween-20, the membranes were probed with the primary polyclonal rabbit antibodies specific for phosphorylated ERK 1/2 (New England Biolabs) at the 1:1000 dilution in the primary antibody dilution buffer (5% bovine serum albumin in TBS/0.1% tween-20), and incubated overnight at 4^{o} C with gentle agitation. The phosphorylated ERK 1/2 antibody recognizes the phosphorylated tyrosine and threonine residues at positions 204 and 202, respectively. The membranes, then, were incubated with horse radish peroxidase conjugated anti-rabbit secondary antibody (Cell Signaling) at a dilution of 1:

2000 in the blocking buffer for 1 hour at room temperature. The protein bands were detected by using LumiGLO™ chemiluminescent detection reagent (New England Biolabs).

To ensure equal loading of protein samples, membranes were probed with polyclonal rabbit IgG actin antibody (Sigma) at a 1:100 dilution using the same protocol as for pERK 1/2. The secondary antibody against the actin IgG was an anti-rabbit IgG coupled to horse radish peroxidase (Cell signaling) at the 1:10,000 dilution.

The membranes were stripped to remove the previously attached antibodies in the stripping buffer (8 ml SDS [2%], 3.125 ml Tris-Cl pH 6.7 [62.5 mM], 350 µl β-mercaptoethanol [100 mM], 38.5 ml deionized water) for 30 minutes to 1 hour in a water bath at 50° C with gentle agitation. The membranes were washed three times in TBS/0.05% tween-20 for 5 minutes each and were stored in cellophane wrap at 4° C.

Densitometric analysis of protein bands:

Densitometric analysis of the autoradiograms was carried out by using UniScan™ gel analysis software. The pixel numbers for every significant changes were determined and placed on the legend of the figures.

Statistical analysis:

Statistical analysis was carried out by using Prism (Graph Pad Software; San Diego, CA). One-way analysis of variance (ANOVA) followed by a Tukey-Kramer multiple comparisons post test was utilized to compare the control groups with the treatment groups. Differences were considered significant if $p < 0.05$.

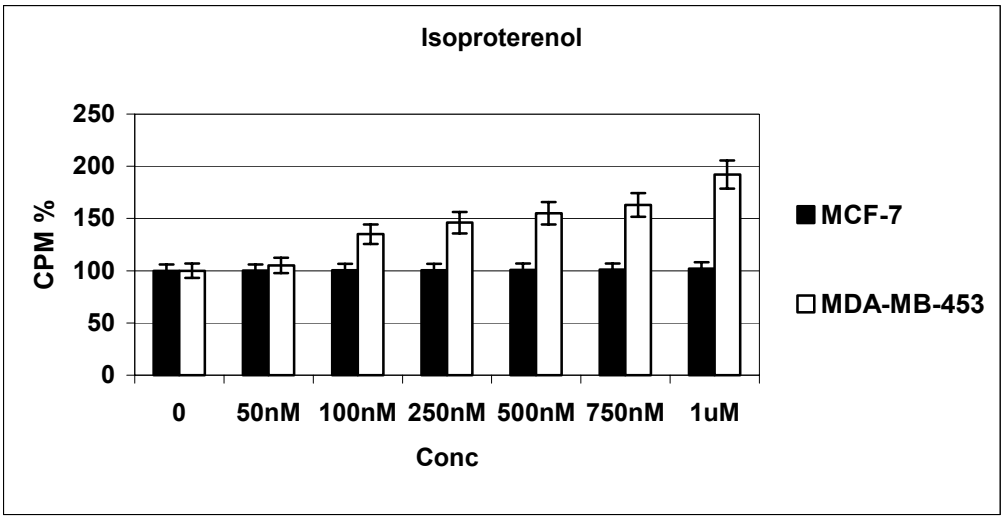
CHAPTER 3: RESULTS

Effects of the beta 1/2 –AR agonist isoprotorenol on human breast cancer cell lines:

Cell proliferation was measured by DNA synthesis as a function of triated thymidine incorporation in all the cell lines studied. The results are demonstrated in figures as bar graphs exhibiting counts per minute (cpm) at different concentrations. The cpm values are exhibited as percentage change in the DNA synthesis for every cell line. Thymidine incorporation studies showed that the β-agonist isoproterenol at concentrations from 100 nM to 1 μM significantly increased DNA synthesis in the ER negative breast cancer cell line, MDA-MB-453 ($p<0.01$) and the increase in the DNA synthesis was almost doubled at the 1 μM concentration ($p<0.01$). By contrast, isoproterenol did not cause any change at these concentrations in any of the ER (+) breast cancer cell lines, MCF-7, ZR-75-1, and MDA-MB-361 (Figure: 8 (a) and (d)).

Isoproterenol only at concentrations from 50 to 250 nM caused a significant DNA synthesis increase in another ER (-) breast cancer cell line, MDA-MB-231 ($p <0.05$) (Figure: 8 (b)). The increase in this cell line was less pronounced than in the other ER (-) cell line, MDA-MB-453. In addition, isoproterenol significantly increased DNA synthesis in the ER (-) MDA-MB-435 breast cancer cell line at concentrations from 50 nM to 1 μM ($p <0.05$) (Figure: 8 (c)). The increases in DNA synthesis were almost identical at every concentration. The only ER (-) breast cancer cell line that did not respond to isoproterenol was MDA-MB-468 (Figure: 8 (b) and 1 (c)). Overall, cell

(a)



(b)

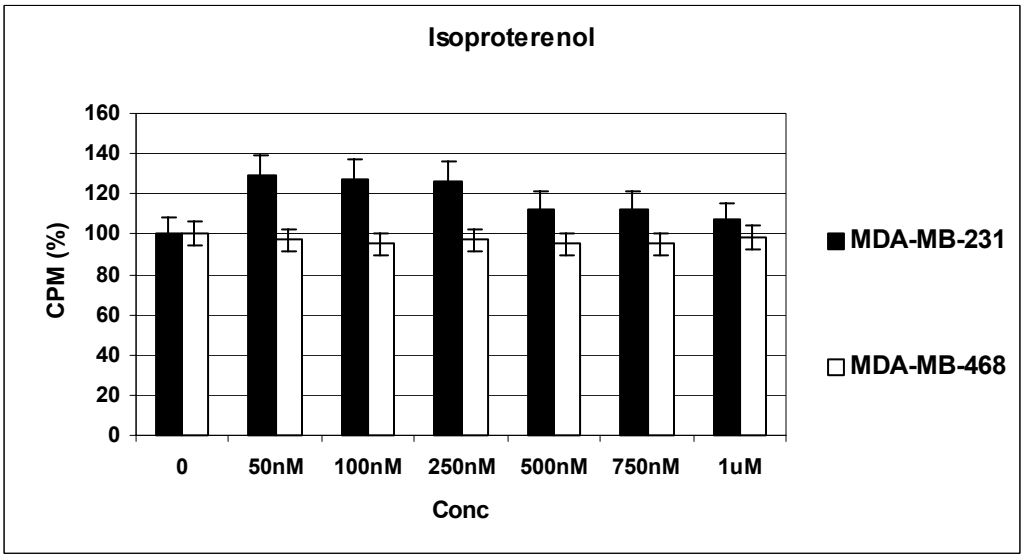
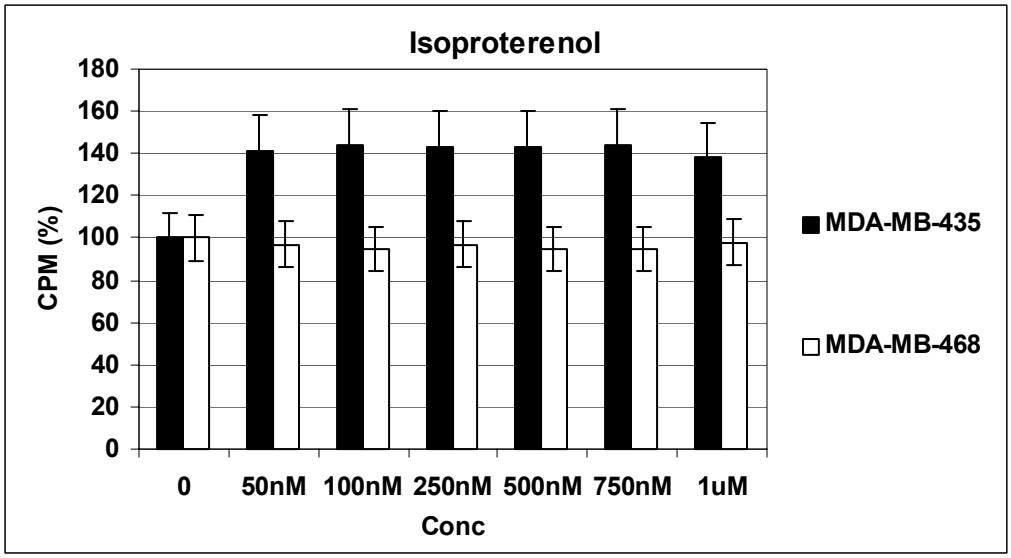


Figure 8: Effects of the general beta 1/2-AR agonist isoproterenol on DNA synthesis. Bars represent the mean and ± SD of each treatment group. Isoproterenol significantly increased DNA synthesis in ER negative breast cancer cell lines, MDA-MB-453 (a), and MDA-MB-231 (b). Isoproterenol did not cause significant increases in DNA synthesis in both ER positive breast cancer cell line MCF –7 and in the ER negative breast cancer cell line MDA-MB-468.

(c)



(d)

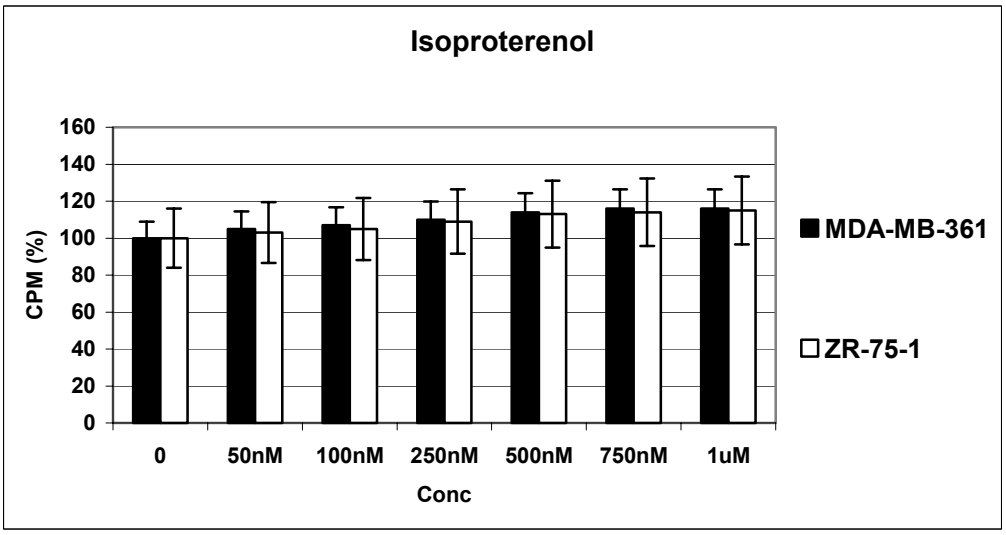


Figure 8: (continued) Effects of the general beta 1/2-AR agonist isoproterenol on DNA synthesis. Bars represent the mean and ± SD of each treatment group. Isoproterenol significantly increased DNA synthesis in ER negative breast cancer cell line, MDA-MB-435 (c). Isoproterenol did not cause significant increases in DNA synthesis in ER positive breast cancer cell lines, MDA-MB-361 and ZR-75-1 (d).

proliferation assay showed that all ER (-) breast cancer cell lines with the exception of one were stimulated in a concentration-dependent manner by isoproterenol.

MAPK activation assays revealed that isoproterenol at the 1 μM concentration caused a slight increase in the activation of ERK 1/2 in the ER (+) cell line MCF-7 at 6 hour, but it declined after 24 hours (Figure: 9). In the ER (-) cell line MDA-MB-453, on the other hand, isoproterenol at the 1 μM concentration slightly increased activation of ERK 1/2 after 24 hour (Figure: 9).

Effects of broad-spectrum β-adrenergic antagonist propranolol on human breast cancer cell lines:

Propranolol decreased DNA synthesis at concentrations from 50 nM to 1 μM in both ER (+) and ER (-) breast cancer cell lines studied. Among the ER (+) breast cancer cell lines, MCF-7 was mostly affected by propranolol with a significant decrease in DNA synthesis at concentrations from 50 nM to 1 μM ($p < 0.01$) (Figure: 10 (a)). Figure: 10 (a) shows that there was a concentration-dependent in DNA synthesis with an almost complete inhibition at the 1 μM concentration. Two other ER (+) breast cancer cell lines, MDA-MB-361 and ZR-75-1 also responded to propranolol with a significant decrease in DNA synthesis ($p < 0.01$) (Figure: 10 (b)). The inhibition of DNA synthesis was concentration-dependent in ZR-75-1 cells, while its effects reversed in MDA-MB-361 cells at the 1 μM concentration.

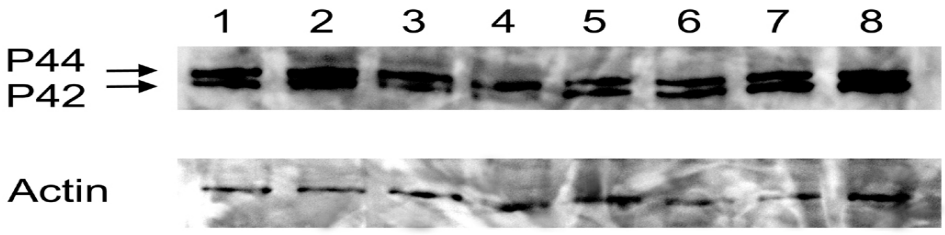
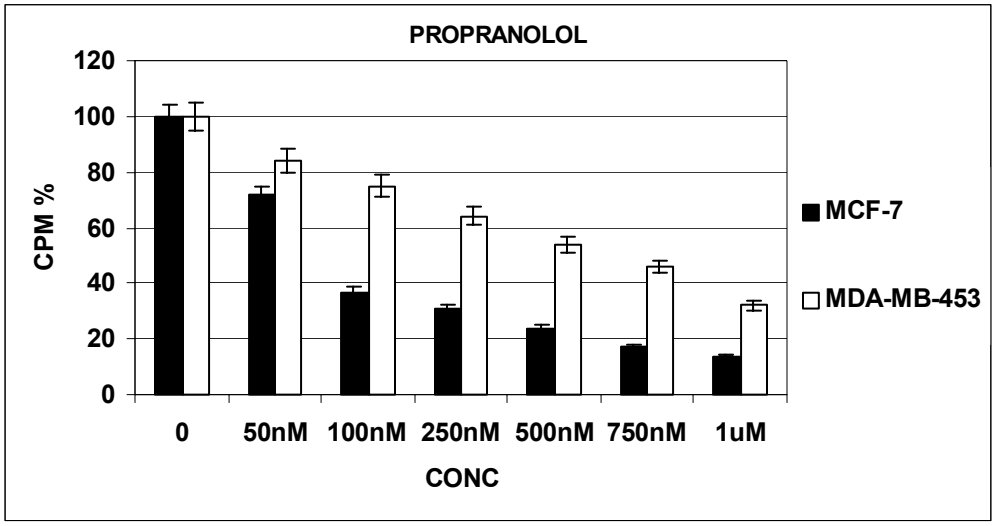


Figure 9**:** Effect of 1 μM isoproterenol on pERK1/2 (p44/ p42) activation in MCF-7 and MDA-MB-453 cell line. Isoproterenol caused a slight increase in the activation of ERK 1/2 in the ER (+) cell line MCF-7 at 6 hour, but it declined after 24 hours. In the ER (-) cell line MDA-MB-453, on the other hand, isoproterenol at the 1 μM concentration slightly increased activation of ERK 1/2 after 24 hour. (Note that parentheses represent densitometry values)

1: MCF-7 6 h- Control (105)
2: MCF-7 6 h- Isoproterenol (135)
3- MCF-7 24 h- Control (132)
4: MCF-7 24 h- Isoproterenol (95)
5: MDA-MB-453 6 h- Control
6: MDA-MB-453 6 h- Isoproterenol
7: MDA-MB-453 24 h- Control (121)
8: MDA-MB-453 24 h- Isoproterenol (158)

(a)



(b)

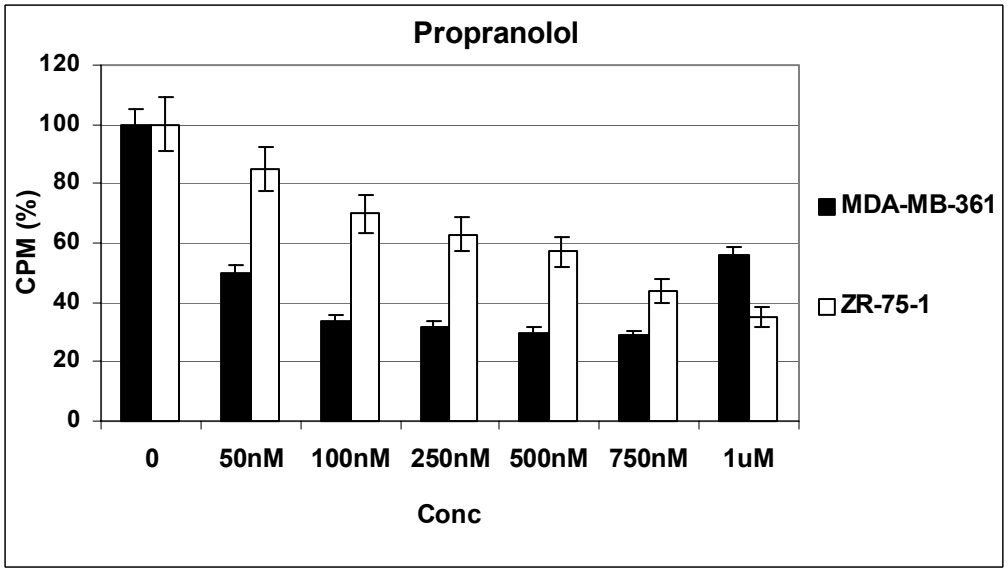
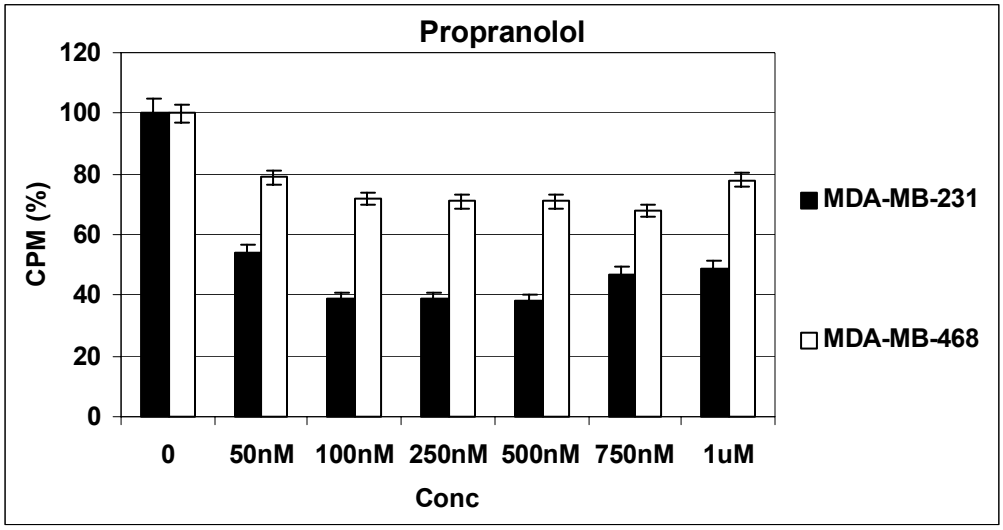


Figure 10: Effect of the broad-spectrum beta-blocker propranolol on DNA synthesis. Bars represent the mean and ± SD of each treatment group. Propranolol significantly decreased DNA synthesis both in ER negative breast cancer cell line, MDA-MB-453 (a), and ER positive breast cancer cell lines, MDA-MB-361 (b), MCF-7 (a) and ZR-75-1 (b). The decrease in MCF-7 cells was more pronounced than in the other cell lines.

(c)



(d)

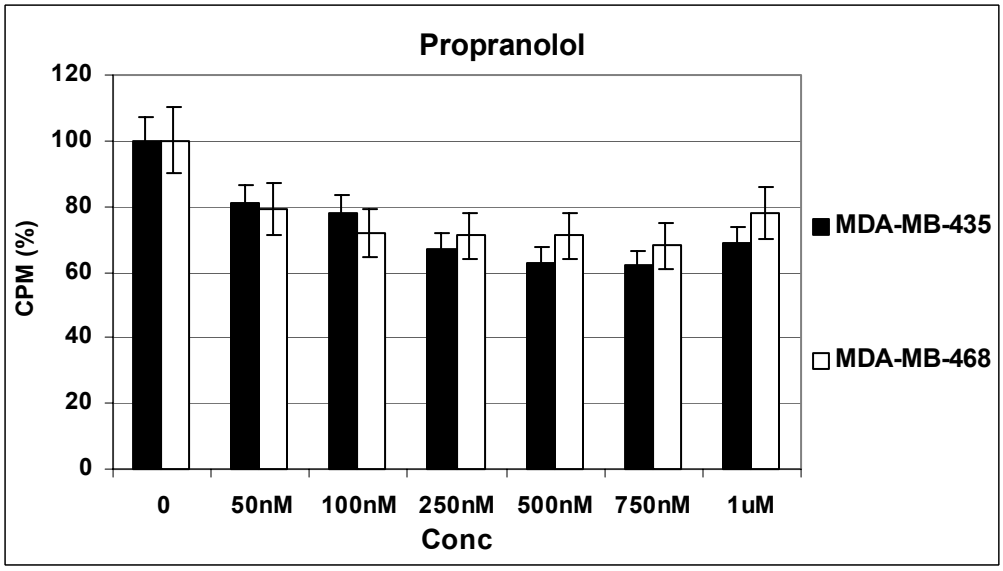


Figure 10: (continued) Effect of the broad-spectrum beta-blocker propranolol on DNA synthesis. Bars represent the mean and ± SD of each treatment group. Propranolol significantly decreased DNA synthesis in ER negative breast cancer cell lines, MDA-MB-468, MDA-MB-231 (c) and MDA-MB-435 (d). The decrease in MDA-MB-231 cell line was more pronounced than in the other cell lines.

Among the ER (-) breast cancer cell lines, DNA synthesis was most significantly reduced in MDA-MB-453 and MDA-MB-231 cell lines ($p < 0.01$) (Figure: 10 (a) and (c)). There was an almost 70 % decrease in DNA synthesis in MDA-MB-453 cells and a 50 % inhibition in MDA-MB-231cells at the 1 μM concentration. In the remaining ER (-) breast cancer cell lines MDA-MB-435 and MDA-MB-468 inhibitions of DNA synthesis were about 35 % at the 750 nM concentration and 25 % at the 1 μM concentration ($p < 0.05$) (Figure: 10 (c) and (d)). Overall, cell proliferation assays demonstrated that the ER (+) breast cancer cell lines were significantly more responsive to inhibitory effects of propranolol.

MAPK expression assays showed that propranolol at the 1 μM concentration reduced the expression of ERK 1/2 in the ER (+) cell line MCF-7 at only 150 minute (Figure: 11). In the ER (-) cell line MDA-MB-453 propranolol (1 μM) reduced the expression of ERK 1/2 at 60 and 150 minutes (Figure: 11). Propranolol did not cause any changes in the activation of ERK 1/2 in both ER (+) and ER (-) breast cancer cell lines, MCF-7 and MDA-MB-453, respectively (Figures: 12 and 13). Overall, these results suggest that propranolol decreased total MAPK protein expression on the DNA level without changing the phosphorylation status of MAPK.

In the RTPCR experiments, continuous exposure to 1 μM propranolol for 7 days increased GIRK1 expression in the ER (-) breast cancer cell line, MDA-MB-453 (Figure: 14 (a)). However, when MDA-MB-453 cells were treated with 1 μM propranolol 30 minutes every day for 7 days, the GIRK1 expression did not change (Figure: 14 (b)).

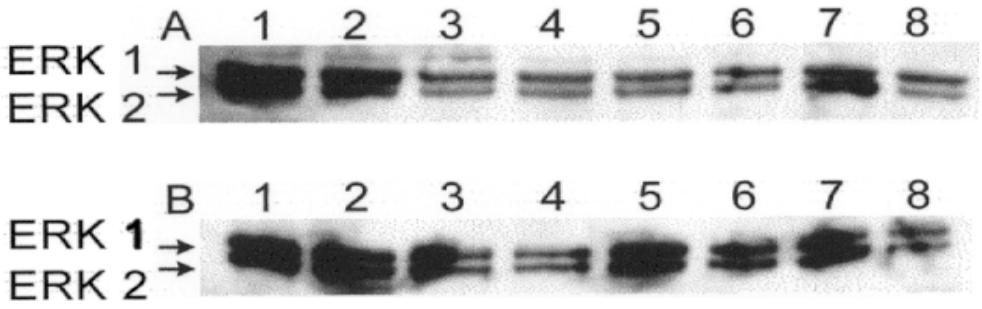


Figure 11: Effect of 1 μM propranolol on ERK 1/2 expression in two breast cancer cell lines. Propranolol decreased the expression of ERK 1/2 in the ER (+) cell line MCF-7 at only 150 minute (Pixel values: 90 / 75). In the ER (-) cell line MDA-MB-453 propranolol decreased the expression of ERK 1/2 at 60 and 150 minutes. (Pixel values: 116 / 78 for 60 min; 116 / 43 for 150 min.)

A: MCF-7 –ER (+)
B: MDA-MB-453- ER (-)

1: 0 min - Control	5: 60 min- -Control
2: 0 min - Propranolol	6: 60 min- Propranolol
3: 15 min - Control	7: 150 min --Control
4: 15 min- Propranolol	8: 150 min- Propranolol

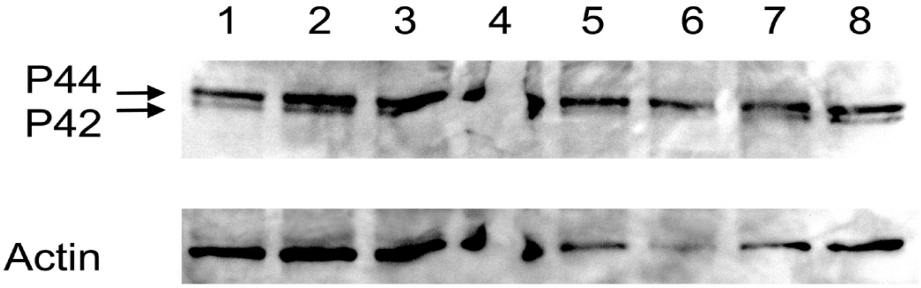


Figure 12: Effect of 1 μM propranolol on pERK1/2 (p44/ p42) activation in the ER (+) MCF-7 cell line. There was no change in the activation status of ERK 1/2.

1: 0 min- Control
2: 0 min- Propranolol
3: 15 min - Control
4: 15 min- Propranolol
5: 60 min- Control
6: 60 min- Propranolol
7: 150 min - Control
8: 150 min- Propranolol

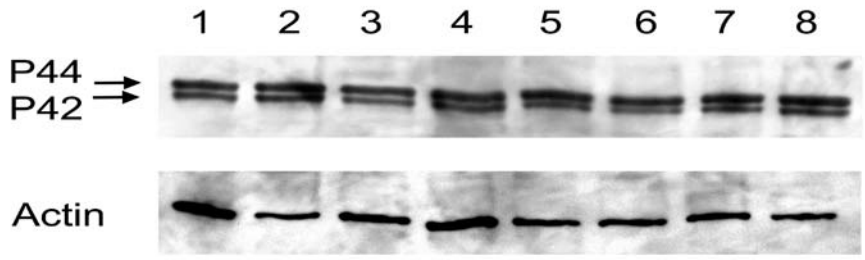
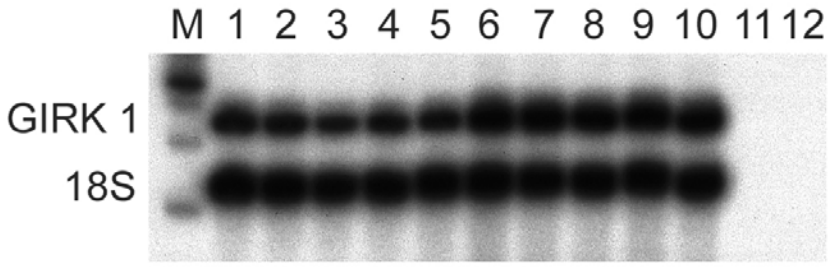


Figure 13: Effect of 1 μM propranolol on pERK1/2 (p44/p42) activation in the ER (-) MDA-MB-453 cell line. There was no change in the activation status of ERK 1/2.

1: 0 min- Control
2: 0 min- Propranolol
3: 15 min - Control
4: 15 min- Propranolol
5: 60 min- Control
6: 60 min- Propranolol
7: 150 min - Control
8: 150 min- Propranolol

(a)



(b)

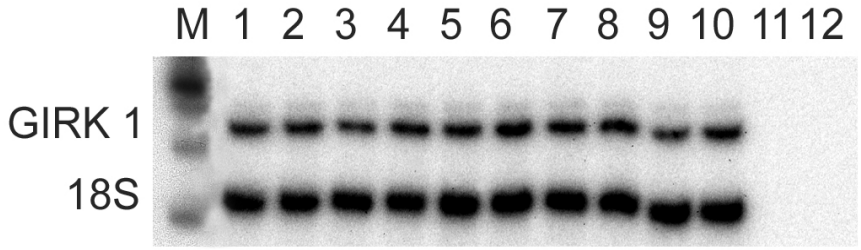


Figure 14: Effect of 1 μM propranolol on GIRK1 expression in ER (-) MDA-MB-453 breast cancer cells. GIRK1 was over-expressed when the cells were continuously exposed to propranolol for 7 days (pixel values: 0.55 / 0.90) (a). GIRK1 expression did not change when the cells were exposed to propranolol for 30min per day for 1 week (b).

1-5: Control
6-10: Propranolol
11-12: Negative Control

These results suggest a possible link between GIRK1 channel activity and β-AR signaling and indicate that up-regulation in channel expression in response to chronic continuous exposure to propranolol was caused by the well-documented antagonist-induced up-regulation of β-adrenergic receptors.

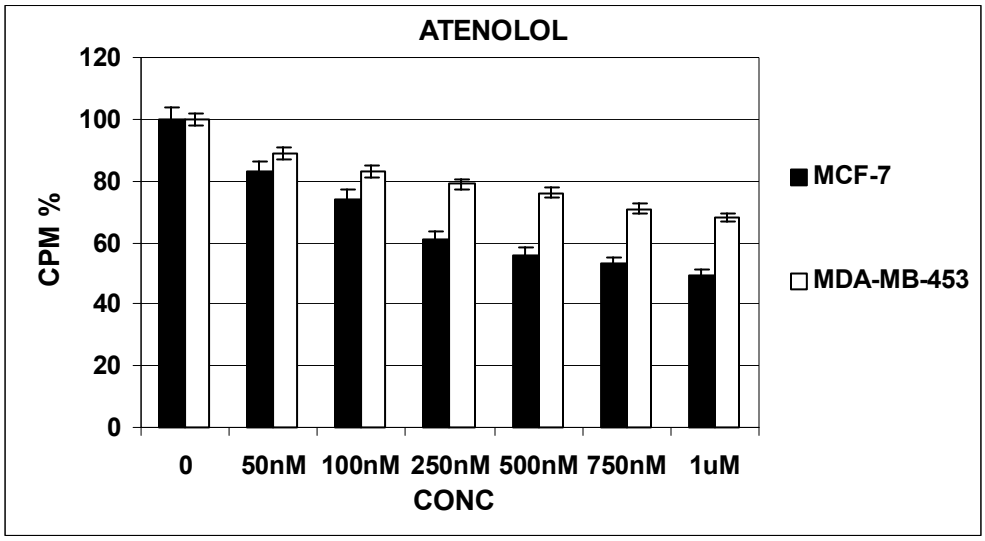
Effects of the selective β_1-AR antagonist atenolol on human breast cancer cell lines:

The ER (+) cell line MCF-7 was the most responsive to atenolol treatment with a 50 % reduction of DNA synthesis below basal level ($p<0.01$) (Figure: 15 (a)). The inhibition was concentration-dependent and reached a maximum at the 1 μM concentration. The ER (+) breast cancer cell lines MDA-MB-361 and ZR-75-1 exhibited almost no inhibition in response to atenolol with only about 20 % reduction of DNA synthesis below controls at each concentration (Figure: 15 (b)).

ER (-) breast cancer cell lines, generally, were not much affected by increasing concentrations of atenolol (Figures: 15 (a), (c) and (d)). Only MDA-MB-468 cell s exhibited a significant inhibition of DNA synthesis (about 45 % below controls; $p <0.05$; Figure: 15 (c) and (d)). On the other hand, DNA synthesis decreased by about 30 % in MDA-MB-453 (Figure: 8 (a)) and MDA-MB-231 cells (Figure: 8 (c)), while it was about 20 % in MDA-MB-435 cells (Figure: 15 (d)). Taken together, only MCF-7 [ER (+)] and MDA-MB-468 [ER (-)] breast cancer cell lines demonstrated a significant response to atenolol.

Atenolol at the 1 μM concentration did not cause any significant changes in the expression of ERK 1/2 in either MCF-7 (Figure: 16 (a)) or MDA-MB-453 (Figure: 16 (b)) breast cancer cells.

(a)



(b)

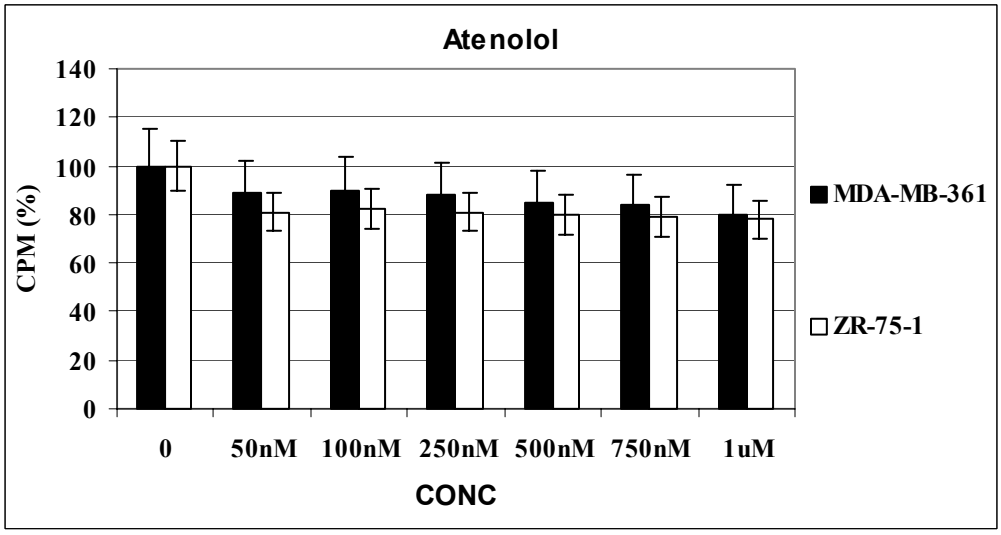
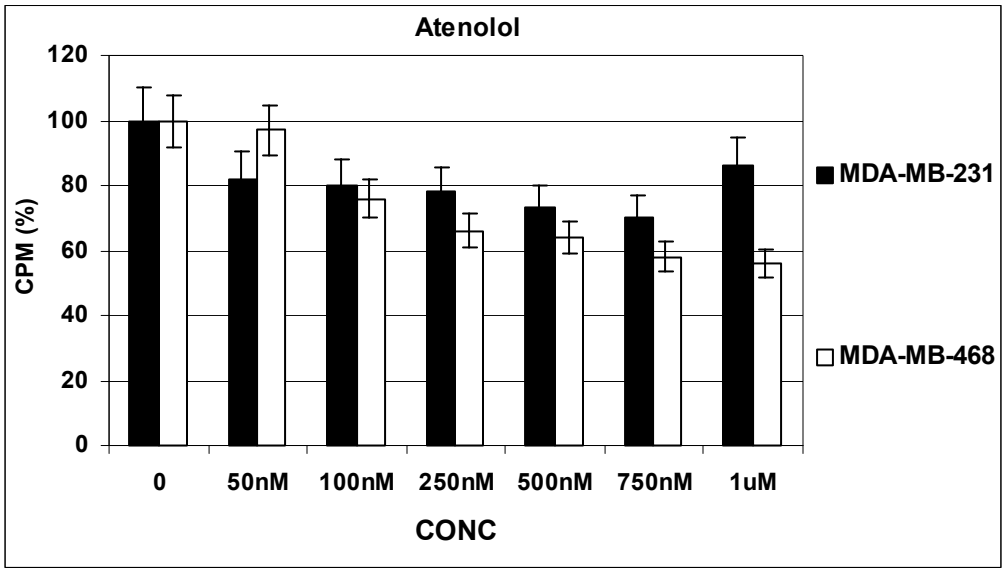


Figure 15: Effect of increasing concentrations of the selective β_1-AR antagonist atenolol on DNA synthesis. Bars represent the mean and ± SD of each treatment group. Atenolol significantly decreased DNA synthesis in the ER positive breast cancer cell line, MCF-7 (a), and in the ER negative breast cancer cell line, MDA-MB-453 (a). MCF-7 cells line were most responsive to atenolol treatment. Two ER positive cell lines exhibited no significant response to atenolol (b).

(c)



(d)

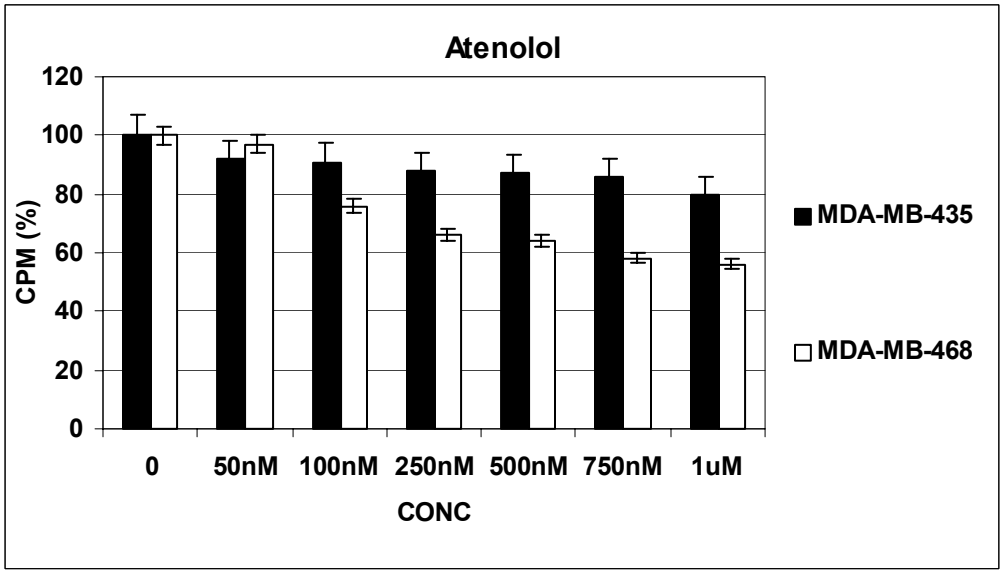
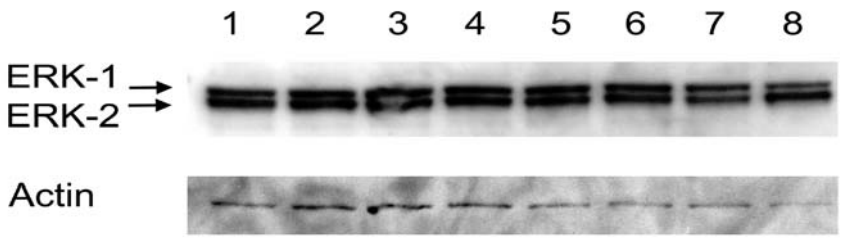


Figure 15: (continued). Effect of increasing concentrations of the selective β_1-AR antagonist atenolol on DNA synthesis. Bars represent the mean and ± SD of each treatment group. Atenolol significantly decreased DNA synthesis in the ER negative breast cancer cell line, MDA-MB-468 (c, d). Two other ER negative cell lines exhibited no significant inhibition in response to atenolol (c, d).

(a)



(b)

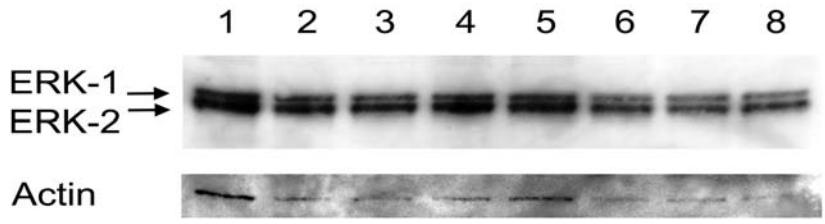


Figure 16: Effect of 1 μM atenolol on ERK1/2 expression in ER (+) MCF-7 (a) and ER (-) MDA-MB-453 (b) cell lines. There was no change in the expression status of ERK 1/2.

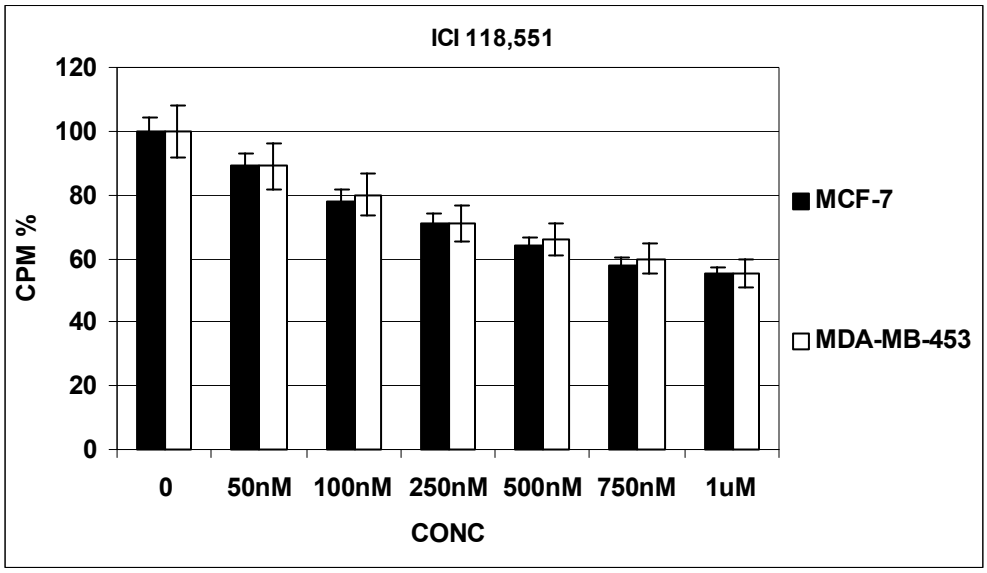
1: 0 min- Control	5: 60 min- Control
2: 0 min- Atenolol	6: 60 min- Atenolol
3: 15 min - Control	7: 150 min- Control
4: 15 min- Atenolol	8: 150 min- Atenolol

Effect of the selective β_2-AR antagonist ICI 118,551 on human breast cancer cell lines:

The ER (+) breast cancer cell lines with the exception of ZR-75-1 significantly responded to increasing concentrations of the β_2-AR antagonist ICI 118-551. The inhibition of DNA synthesis in MCF-7 cell line was about 45 % below that of controls ($p<0.05$) (Figure: 17 (a)). The most dramatic response to ICI 118,551 was observed in MDA-MB-361 cell with an 80 % decrease in the DNA synthesis at 500 nM, 75 % at 750 nM and 60 % at the 1 μM concentration ($p< 0.01$) (Figure: 17 (b)). In cell line ZR-75-1, a significant 40 % inhibition in DNA synthesis was observed only at a 1 μM concentration ($p<0.05$) (Figure: 17 (b)).

Similar to the MCF-7 cell line, the ER (-) breast cancer cell line MDA-MB-453 showed only a 45 % reduction in DNA synthesis ($p < 0.05$) (Figure: 17 (a)), whereas MDA-MB-231 cells exhibited a concentration-dependent response with a 65 % inhibition at 500 nM ($p <0.01$), 60 % at 750 nM ($p < 0.01$) and 30 % at 1 μM concentration ($p < 0.05$) (Figure: 17 (c)). The other two ER (-) cell lines reacted in a similar manner. At 500 and 750 nM ICI 118, 551 concentrations, the reductions in DNA synthesis were almost 40 % below control ($p <0.05$) even though the decrease reversed at the 1 μM concentration being 35 % and 22 % for the MDA-MB-468 and MDA-MB-435, respectively (Figure: 17 (c) and (d)). Overall, the response of the ER (+) cell line MDA-MB-361 and the ER (-) cell line MDA-MB-23 to ICI 118, 551 was significantly more pronounced than the response of other cell lines.

(a)



(b)

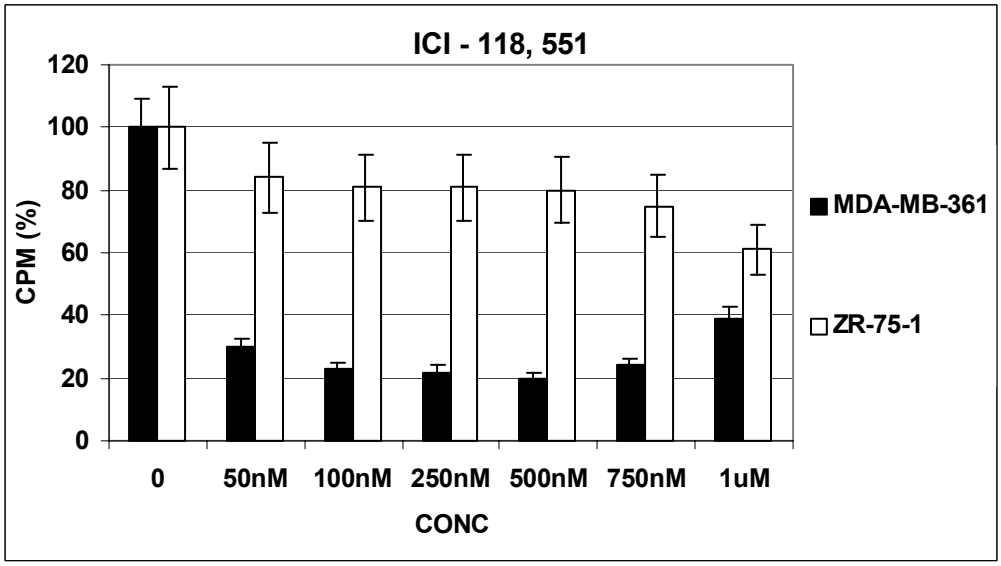
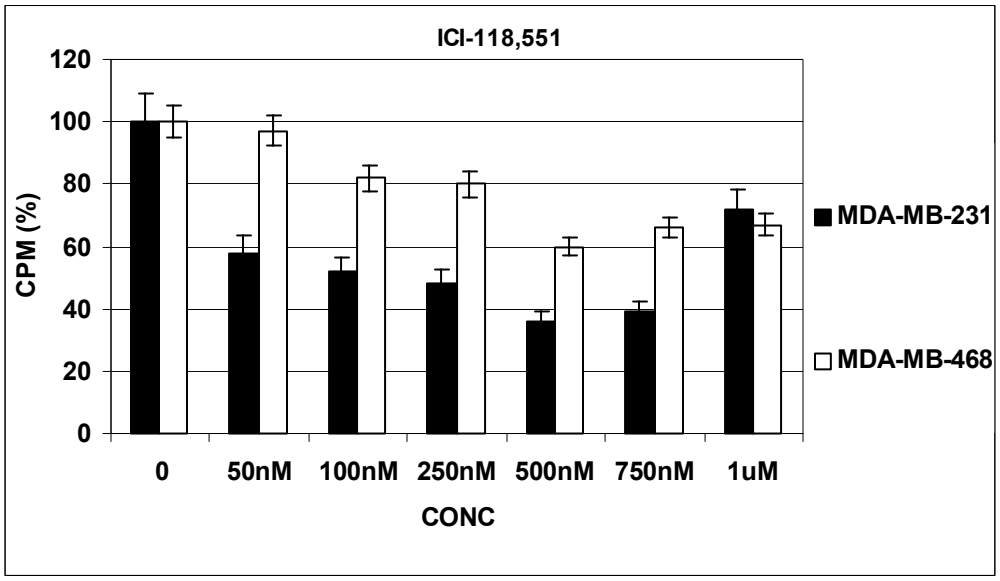


Figure 17: Effect of increasing concentrations of selective β_2-AR antagonist ICI 118,551 on DNA synthesis. Bars represent the mean and ± SD of each treatment group. ICI 118,551 significantly decreased DNA synthesis in ER positive breast cancer cell lines, MCF-7 and MDA-MB-361 (b), and ER negative breast cancer cell line, MDA-MB-453 (a). MDA-MB-361 was the most responsive to atenolol treatment though in ZR-75-1 significant inhibition was seen only at 1 uM concentration.

(c)



(d)

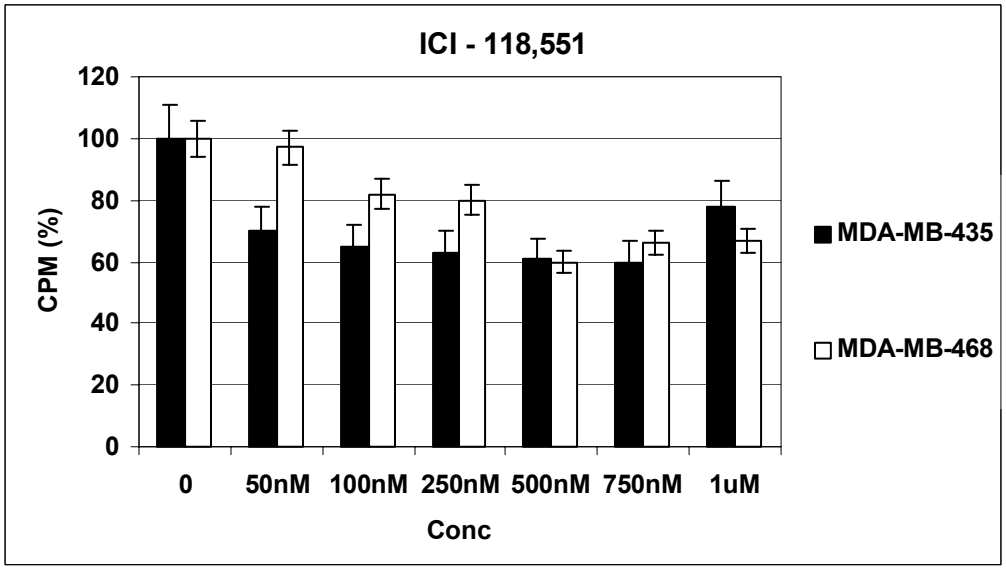
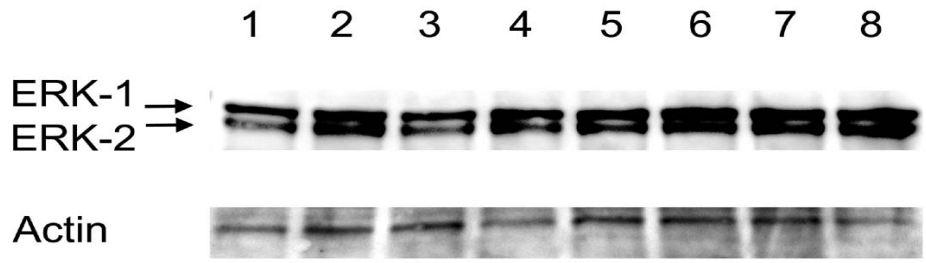


Figure 17: (continued) Effect of increasing concentrations of the selective β_2-AR antagonist ICI 118,551 on DNA synthesis. Bars represent the mean and ± SD of each treatment group. ICI 118,551 significantly decreased DNA synthesis in ER negative breast cancer cell lines, MDA-MB-231 (c), MDA-MB- 435 (d) and MDA-MB-468 (d). The MDA-MB-231 cell line was much more affected by ICI 118, 551 treatments than other cell The ER (+) MCF-7 cell line did not show any difference in the expression status of lines.

(a)



(b)

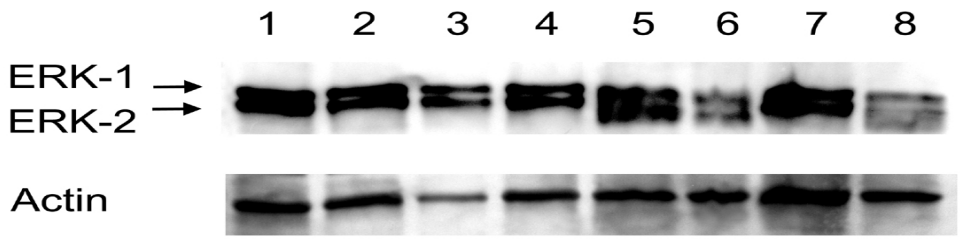


Figure 18: Effect of 1 μM ICI 118,551 on ERK1/2 expression in ER (+) MCF-7 (a) and ER (-) MDA-MB-453 (b) cell lines. There was no change in the expression status of ERK 1/2 in MCF-7 (a) whereas ICI 118,551 reduced the expression of ERK 1/2 at 60 and 150 minutes in MDA-MB-453 breast cancer cell line (pixel values: 277 / 126 for 60 min; 269 / 132 for 150 min.) (b).

1: 0 min-	Control
2: 0 min-	ICI 118,551
3: 15 min -	Control
4: 15 min-	ICI 118,551
5: 60 min-	Control
6: 60 min-	ICI 118,551
7: 150 min-	Control
8: 150 min-	ICI 118,551

The ER (+) MCF-7 cell line did not show any difference in the expression status of ERK 1/2 (Figure: 18 (a)) while only the ER (-) breast cancer cell line MDA-MB-453 demonstrated a declined ERK 1/2 expression at 60 and 150 minutes in response to 1 μM ICI 118, 551 (Figure: 18 (b)).

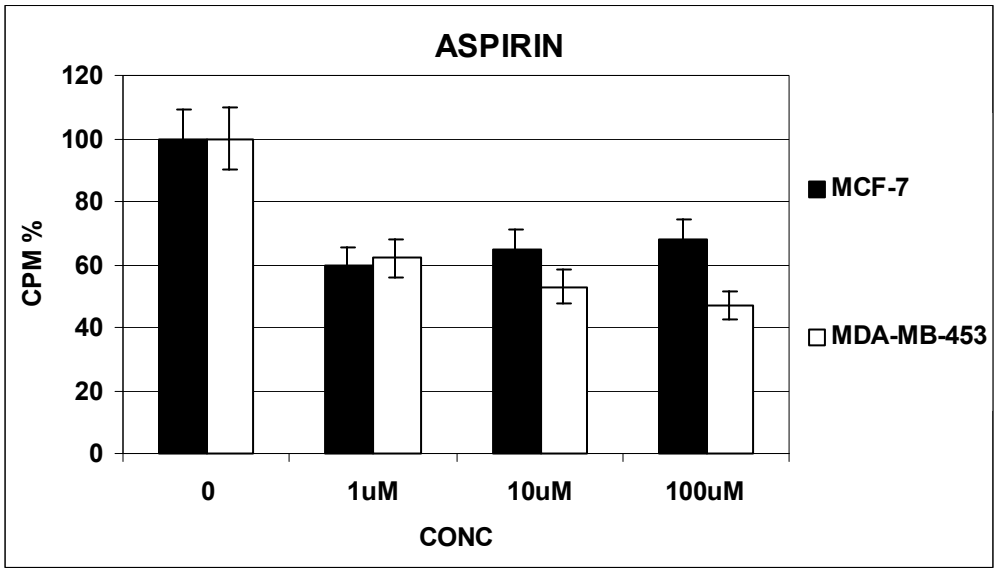
Effects of the COX 1/2 inhibitor aspirin on human breast cancer cell lines:

The COX 1/2 inhibitor aspirin reduced DNA synthesis in the ER (+) cell line, MCF-7 and in the ER (-) cell line MDA-MB-453 (Figure: 19 (a)). Both cell lines responded to aspirin treatment in the same manner at the 1 μM concentration with DNA synthesis being reduced almost 40 % below the controls ($p < 0.05$). However, the MDA-MB-453 cell line continued to be inhibited by increasing concentrations of aspirin ($P < 0.05$), while MCF-7 started to lose its responsiveness to aspirin with inhibition of DNA synthesis decreasing from 40 % to 30 % though it was still significant ($p< 0.05$). These findings suggest that the ER (-) cell line MDA-MB-453 may have higher levels of expression of COX 1/2 than the ER (+) cell line MCF-7.

Effect of the FLAP (5-LOX activating protein) inhibitor MK-886 on human breast cancer cell lines:

The FLAP inhibitor MK-886 reduced DNA synthesis in ER (-) and ER (+) cell lines (Figure: 19 (b)). The decline in DNA synthesis at a concentration of 100 nM concentration of MK-886 was almost identical for both cell lines, being 45 % and 50 % below controls for MCF-7 and MDA-MB-453, respectively ($p < 0.05$). Nonetheless, when the concentrations of MK-886 were increased to 1 and 10 μM, the ER (-) breast

(a)



(b)

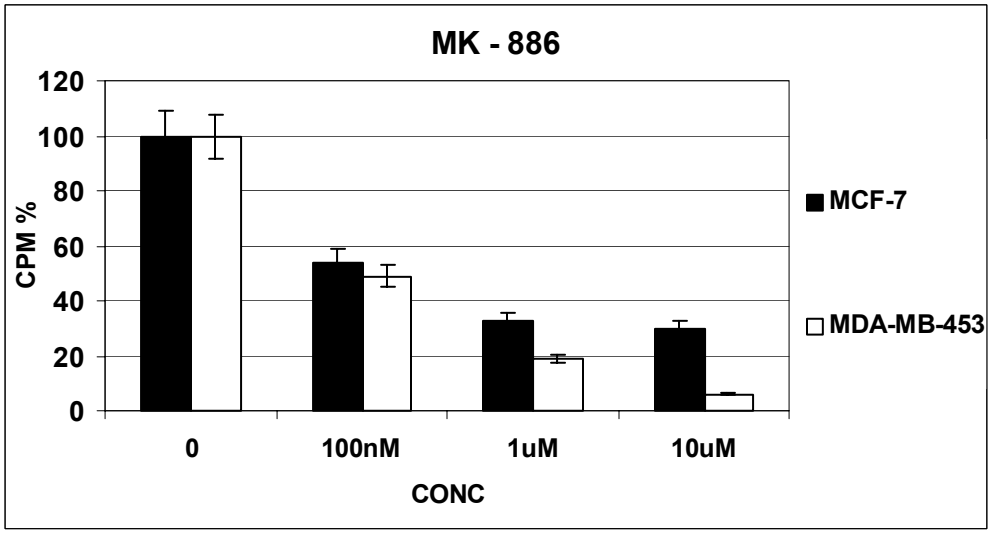


Figure 19: Effects of increasing concentrations of aspirin and MK-886 on DNA synthesis. Bars represent the mean and ± SD of each treatment group. Aspirin significantly decreased DNA synthesis in MCF-7 and MDA-MB-453 (a). Inhibition was more pronounced in MDA-MB-453 than MCF-7. At each concentration, MK-886 treatment resulted in a significant decrease as compared with controls (b). Both cell lines were affected by MK-886. The ER negative cell line MDA-MB-453 was more responsive to the inhibitory action of MK-886.

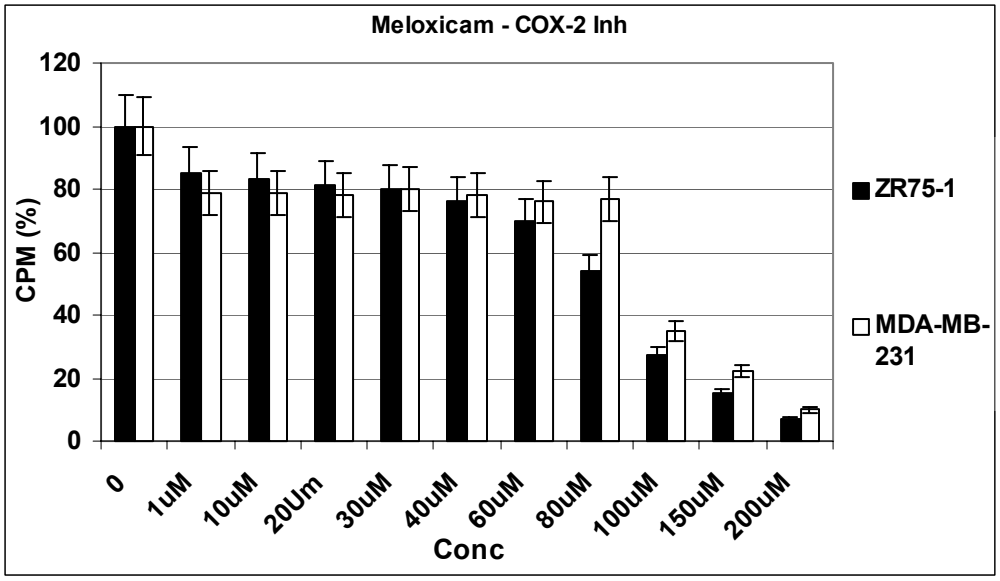
cancer cell line MDA-MB-453 demonstrated a strong reduction in DNA synthesis (80 % at the 1 μM and 95% at the 10 μM; $p < 0.01$) whereas the ER (+) cell line MCF-7 exhibited somewhat less but concentration-dependent reduction in DNA synthesis at these concentrations (68 % at the 1 μM and 70 % at the 10 μM concentration). Similar to the results obtained with aspirin, these findings suggest that the ER (-) cell line MDA-MB-453 may have higher levels of 5-LOX expression than the ER (+) cell line MCF-7.

Effect of the specific COX-2 inhibitor meloxicam on human breast cancer cell lines:

Generally, meloxicam up to 80 μM concentrations caused little decrease in DNA synthesis in the breast cancer cell lines studied. At a concentration of 80 μM, meloxicam, reduced DNA synthesis by about 45 % below controls in the ER (+) cell line ZR-75-1 ($p < 0.001$) (Figure: 20 (a)), whereas the other three ER (-) cell lines responded with only with a reduction in DNA synthesis of about 20 % ($p > 0.05$), % 27 ($p < 0.05$) and 30 % ($p < 0.05$) over time (Figure: 20 (a) and (b)). At a concentration of100 μM, the most pronounced inhibition of DNA synthesis was observed in both ER (-) breast cancer cell lines with MDA-MB-231 showing an about 67 % reduction over time ($p < 0.001$) while the ER (+) breast cancer cell line ZR-75-1 showed an almost 70 % inhibition ($p < 0.001$) (Figure: 20 (a)).

Of the other two ER (-) cell lines, only MDA-MB-468 cells showed a significant inhibition in DNA synthesis 9about 35 % over time $p < 0.01$) at this concentration (Figure: 20 (b)). As it illustrated in figures 20 (a) and 20 (b), all ER (+) and ER (-) cell lines responded with a significant inhibition of DNA synthesis to 150 and 200 μM concentrations of meloxicam ($p < 0.001$). In addition, the ER (+) ZR-75-1 and ER (-)

(a)



(b)

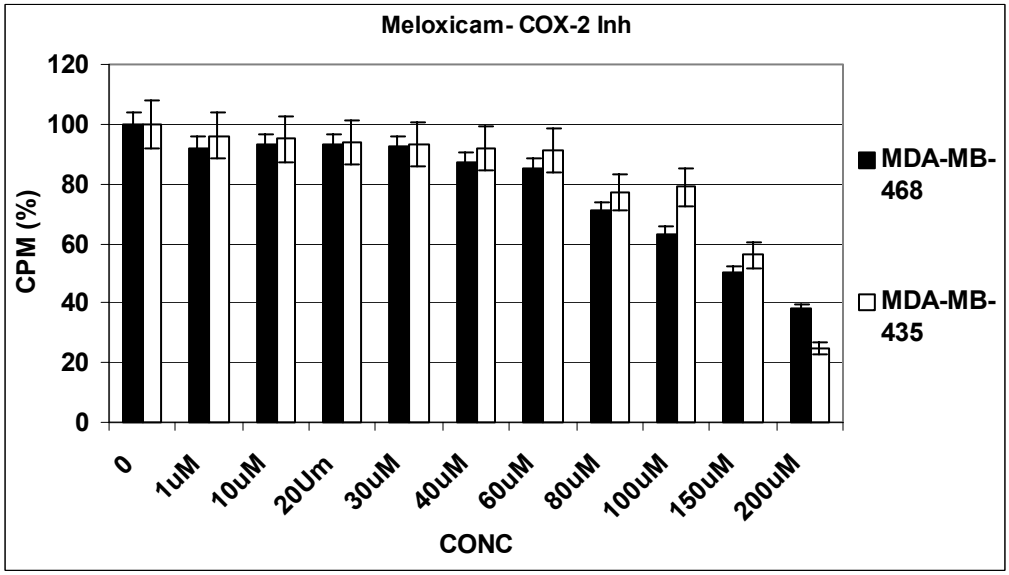


Figure 20: Effect of increasing concentrations of meloxicam on DNA synthesis. Bars represent the mean and ± SD of each treatment group. Meloxicam significantly decreased DNA synthesis in all cell lines at higher concentrations. The ER (-) MDA-MB-231 (a) and ER (+) ZR-75-1 cell lines appeared to be more sensitive to the effect of Meloxicam than the other two ER (-) cell lines (b).

MDA-MB-231 cell lines were more responsive to meloxicam treatment at these concentrations than the other cell lines. Overall, these results suggest that at 100 and 150 μM concentrations the ability of meloxicam to inhibit DNA synthesis was very strong.

MAPK expression studies exhibited that (with the exception of the ER (+) breast cancer cell line MCF-7 and the ER (-) cell line MDA-MB-231) there was no reduction in ERK 1/2 expression in response to 100 μM meloxicam (Figure: 21 (a), (b) and (c)). The expression of ERK 1/2 appeared slightly decreased in MCF-7 cells (Figure: 21 (c), lanes 3 and 4), while only ERK 1 expression was reduced in MDA-MB-231 cells (Figure: 21 (c), lanes 7 and 8) though the status of ERK 2 remained unchanged. The MDA-MB-453 cell line showed an increase in ERK 1/2 expression (Figure: 21 (a), lanes 7 and 8), whereas the other three cell lines, MDA-MB-435, MDA-MB-468 and ZR-75-1 did not exhibit any change in the expression status of ERK 1/2 (Figure: 21 (a) and (b)). These results, suggest that only two ER (-) breast cancer cell lines, MDA-MB-231 and MDA-MB-453, and one ER (+) breast cancer cell line MCF-7 responded with a modulation in ERK 1/2 expression to meloxicam.

Effects of the non-specific 5-LOX inhibitor NDGA on human breast cancer cell lines:

NDGA was used at lower concentrations than meloxicam. In the two ER (-) cell lines, MDA-MB-435 and MDA-MB-468, a significant reduction in DNA synthesis occurred at the higher concentrations (Figure: 22 (a)). The three lowest concentrations (100 nM, 500 nM , 1 μM) did not cause significant changes in DNA synthesis in any of the cell lines studied. However, higher concentrations of NDGA reduced DNA synthesis

(a)

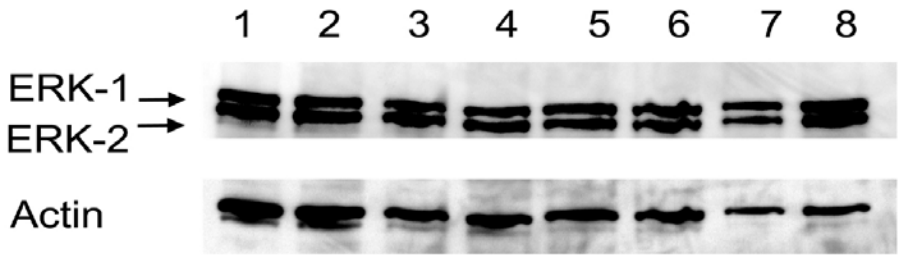


Figure 21: Effect of 30 μM NDGA and 100uM meloxicam on ERK1/2 protein expression in ER (+) ZR-75-1 and ER (-) MDA-MB-453 (a). The MDA-MB-453 cell line showed an increase in ERK 1/2 protein expression (lanes 7 and 8) (pixel values: 90 / 146).

1: ZR-75-1 24 h- Control
2: ZR-75-1 24 h- NDGA
3- ZR-75-1 24 h- Control
4: ZR-75-1 24 h- Meloxicam
5: MDA-MB-453 24 h- Control
6: MDA-MB-453 24 h- NDGA
7: MDA-MB-453 24 h -Control
8: MDA-MB-453 24 h-Meloxicam

(b)

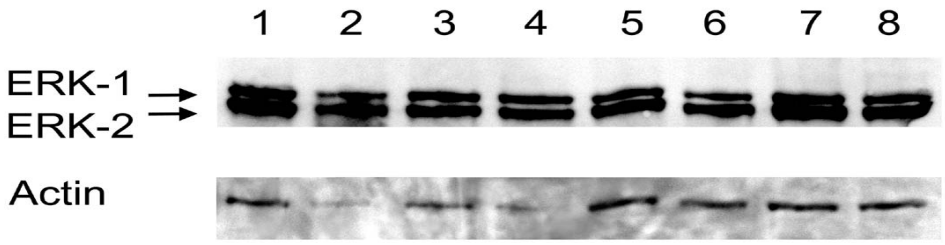


Figure 21: (continued) Effect of 30 μM NDGA and 100uM Meloxicam on ERK1/2 expression in ER (-) MDA-MB-435 and ER (-) MDA-MB-468 cell lines (b).

1: MDA-MB-435 24 h- Control
2: MDA-MB-435 24 h- NDGA
3- MDA-MB-435 24 h- Control
4: MDA-MB-435 24 h- Meloxicam
5: MDA-MB-468 24 h- Control
6: MDA-MB-468 24 h- NDGA
7: MDA-MB-468 24 h -Control
8: MDA-MB-468 24 h- Meloxicam

(c)

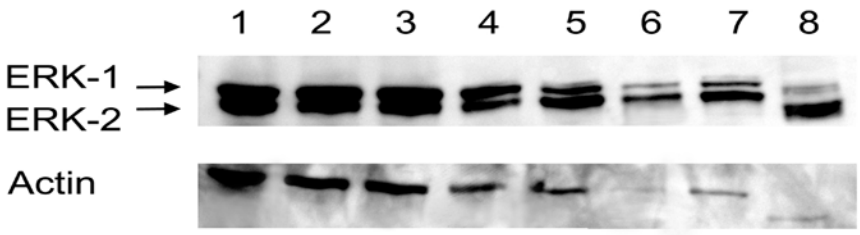
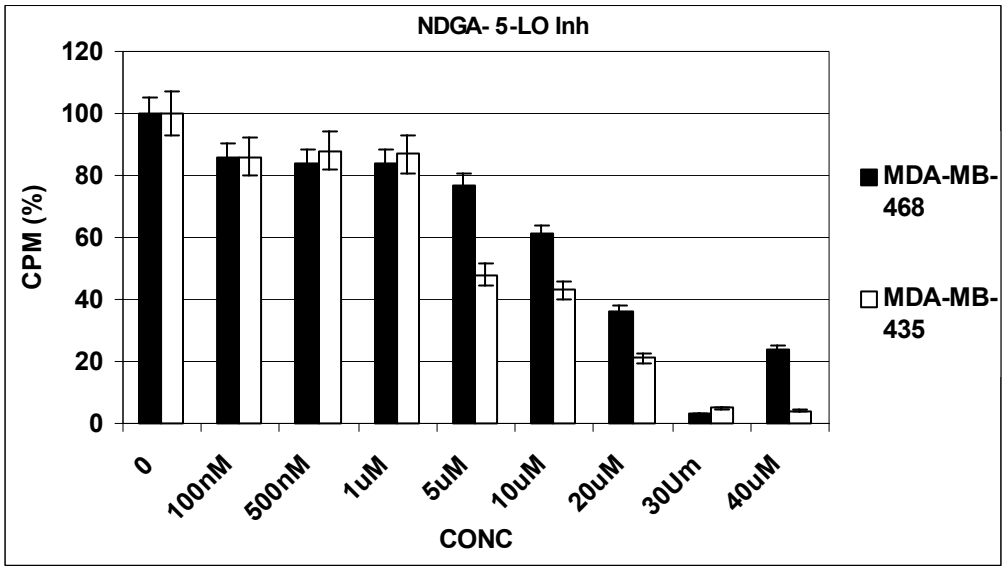


Figure 21: (continued) Effect of 30 μM NDGA and 100uM Meloxicam on ERK1/2 protein expression in ER (+) MCF-7 and ER (-) MDA-MB-231 cell lines. The expression of ERK 1/2 protein appeared slightly decreased in MCF-7 cells (lanes 3 and 4), while only ERK 1 protein expression was reduced in MDA-MB-231 cells (lanes 7 and 8), though the status of ERK 2 expression remained unchanged in MDA-MB-231 cell line.

1: MCF-7 24 h- Control
2: MCF-7 24 h- NDGA
3- MCF-7 24 h- Control
4: MCF-7 24 h- Meloxicam
5: MDA-MB-231 24 h- Control
6: MDA-MB-231 24 h- NDGA
7: MDA-MB-231 24 h -Control
8: MDA-MB-231 24 h-Meloxicam.

(a)



(b)

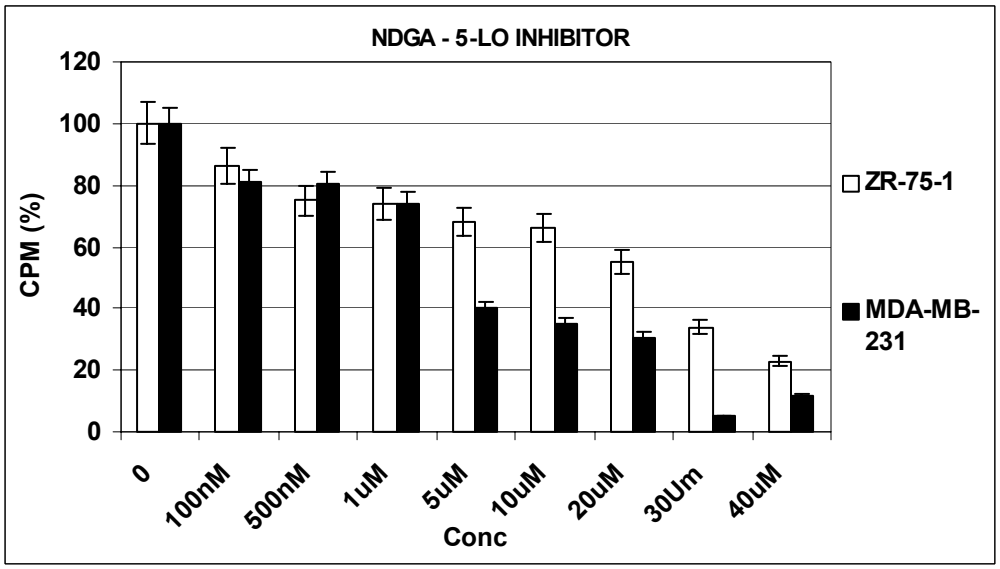


Figure 22: Effect of increasing concentrations of NDGA on DNA synthesis. Bars represent the mean and ± SD of each treatment group. NDGA reduced DNA synthesis in all cell lines. At higher concentrations, three ER (-) cell lines MDA-MB-435 (a), MDA-MB-468 (a) and MDA-MB-231 (b) were more responsive to NDGA treatment than the ER (+) cell line ZR-75-1 (b).

significantly in these two ER (-) breast cancer cell lines. The reductions were 25 % at 5 μM ($p < 0.05$), 38 % at 10 μM ($p < 0.001$), 62 % at 20 μM ($p < 0.001$), 95 % at 30 μM ($p < 0.001$) and 78 % at 40 μM ($p < 0.001$) of NDGA in MDA-MB-468 breast cancer cells (Figure: 22 (a)). The other ER (-) cell line, MDA-MB-435, demonstrated a concentration-dependent decrease in DNA synthesis from 50 to 97% below controls ($p<0.001$) in response to concentrations of NDGA from 5 μM to 40 μM.

The lowest concentration of NDGA (100 nM) only reduced DNA synthesis significantly (20%, $p<0.05$) in the ER (-) breast cancer cell line MDA-MB-231 (Figure: 22 (b)). The higher NDGA concentrations up to 40 μM caused significant reductions in DNA synthesis from 25 % to 90 % over time in this cell line ($p < 0.001$). NDGA concentrations from 500 nM to 40 μM decreased DNA synthesis in the ER (+) breast cancer cell line ZR-75-1 ($p < 0.001$) (Figure: 22 (b)), whereas the lowest concentration (100 nM) exhibited no significant inhibition in this cell line. Overall, these results suggest that NDGA was more effective in inhibiting DNA synthesis at lower concentrations, and all three ER (-) cell lines (MDA-MB-435, MDA-MB-468 and MDA-MB-231) were more responsive to NDGA treatment than the ER (+) cell line ZR-75-1.

Reductions in ERK 1/2 expression in response to NDGA were only observed in the three ER (-) breast cancer cell lines (MDA-MB-435, MDA-MB-468 and MDA-MB-231 (Figure: 21 (a), (b) and (c)). NDGA at 30 μM decreased the expression of MAPK (ERK 1/2) in both MDA-MB-435 (Figure: 21 (b), lanes 1 and 2), and MDA-MB-231 cells (Figure: 21 (c), lanes 5 and 6), while only ERK 1 expression was decreased in MDA-MB-468 (Figure: 21 (b), lanes 5 and 6). In summary, NDGA only caused

significant inhibition of MAPK expression in ER (-) breast cancer cell lines.

Effects of the K^{+1} channel inhibitor quinidine on human breast cancer cell lines:

Quinidine significantly reduced DNA synthesis in both ER (+) MCF-7 and ER (-) MDA-MB-453 breast cancer cell lines (Figure: 23). The reduction was more pronounced in MDA-MB-453 than MCF-7. DNA synthesis in MDA-MB-453 cells was significantly decreased at each concentration of quinidine tested. In MCF-7 cells the lowest concentration of quinidine (10 μM) did not inhibit DNA synthesis whereas all higher concentrations caused significant inhibitions. In both cell lines, the decline in DNA synthesis was dependent-dependent (30 % at 10 μM ($p < 0.05$), 60 % at 40 μM ($p < 0.001$) and 81 % at 120 μM ($p < 0.001$) for MDA –MB-453; and 15 % at 10μM ($p > 0.05$), 48 % at 60 μM ($p < 0.001$) and 70 % at 120 μM for the MCF-7 breast cancer cell line. These result suggests that quinidine was an effective inhibitor for both ER (+) and ER (-) breast cancer cell lines and that ER (-) cell line much more sensitive to this effect.

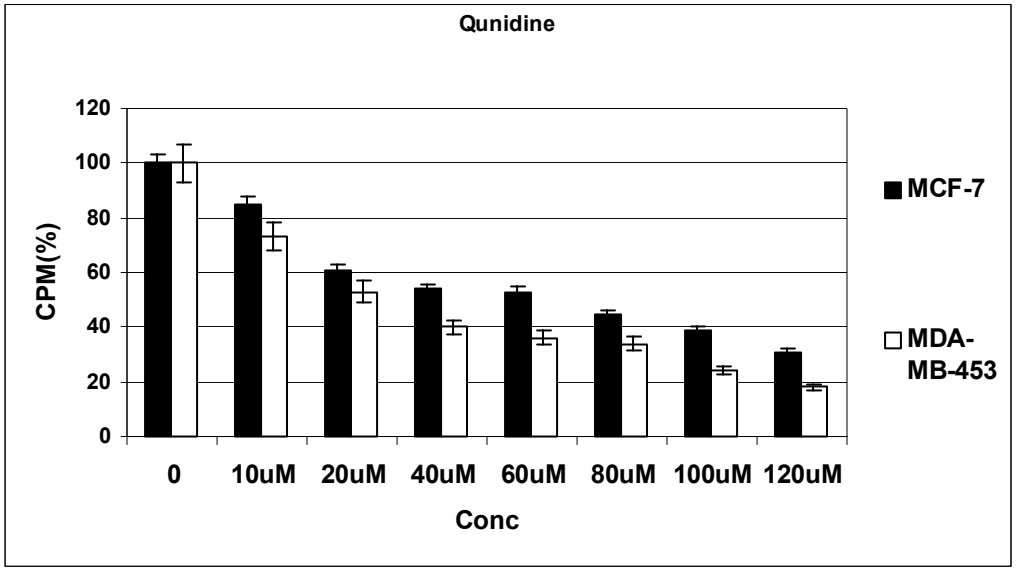


Figure 23: Effect of increasing concentrations of quinidine on DNA synthesis. Bars represent the mean and ± SD of each treatment group. Quinidine significantly reduced DNA synthesis in both ER (+) MCF-7 and ER (-) MDA-MB-453 breast cancer cell lines. The inhibition in the DNA synthesis was more pronounced in MDA-MB-453 cell line than MCF-7.

CHAPTER 4: DISCUSSION

Breast cancer is the most common cancer in the world with at least one million new cases each year and comprises 18 % of all female cancers (132). Even though surgery is the major curative action to remove the primary cancerous lesion, frequent relapses at different sites due to micrometastases is a serious problem that often kills the patient. Conventional cancer therapy often causes undesirable side effects because it is not selective for all cancer cells. Improvements in the understanding of the molecular mechanisms of breast cancer development either genetically or epigenetically have led to many new therapeutic developments that may inhibit invasive breast cancer progression. The latest approaches for the prevention of breast cancer at the molecular level include the administration of newly developed monoclonal antibodies against human epidermal growth factor receptor-2 (HER-2) and receptor tyrosine kinases (RTKs), specific kinase inhibitors, ER antagonists, cell cycle kinase inhibitors, histone deacetylase inhibitors to suppress gene expressions, angiogenesis inhibitors, metalloproteinase inhibitors, new cytotoxic agents and radiation called adjuvant therapy, selective estrogen receptor modulators (raloxifene), apoptosis inducers, telomerase inhibitors, dietary modulators and vitamins, NSAIDs and specific COX-2 inhibitors, and smart drugs targeting only tumor cells and sparing the normal cells (133).

The growth of breast cancer cells is regulated by many factors, including steroid hormones and peptide growth factors, which induce different intracellular signaling pathways. The growth factors bind to tyrosine kinase-associated transmembrane receptors so that they can modulate the other signaling pathways to induce the expression

of transcription factors, leading to the growth and proliferation of tumor cells. Estrogen and several growth factors interact with each other, by co-operating through their respective pathways in the regulation of the proliferation and progression of ER (+) breast tumors (134), while in the ER (-) tumors, the growth factors, particularly EGF, without estrogen induces the downstream signaling pathways when it binds to its receptor (54,58,135-139).

The critical mitogen in many breast cancers is the steroid hormone estrogen even though certain growth factors also play a crucial role in modulating mammary gland development and breast cancer progression. In addition to cell growth and proliferation mediated by estrogen in breast carcinogenesis, mutations in critical genes are often needed for cancer progression (42,134,140). Increased ER expression in most breast tumors is associated with a possible ER gene amplification, stabilization of the ER transcript and increased efficiency of translation of ER protein. The ER loss in the ER (-) tumors is associated with factors effecting the efficient translation of the ER protein. These factors may include ER gene mutation, generation of different ER variants, aberrant hypermethylation of ER CpG island in the regulatory region of the ER gene, inactivation of the co-activators or over-expression of co-repressor and the loss of transcription factor ERF-1 that are required for enhanced ER gene expression (42, 141, 142).

Real time polymerase chain reaction (PCR) studies by Iwao et al. demonstrated that in the ER (+) tumors, ERα mRNA levels were much higher while in the ER (-) tumors, ERβ mRNA levels were higher (143). Furthermore, ERβ / ERα ratio was higher in the ER (-) tumors than ER (+) tumors , suggesting that the quantitative analysis of ERα

and β by the real time PCR corresponds to the analysis of these receptors by the conventional RT-PCR. Similarly, it was also demonstrated by the real time PCR that the ERα was over-expressed in the PR (+) tumors whereas the ERβ levels were higher in the PR (-) tumors (143).

We conducted cell proliferation assays using the incorporation of [^{3}H]-thymidine to monitor DNA synthesis. Our studies demonstrate that the broad-spectrum β-adrenergic antagonist propranolol significantly reduced cell growth in all cell lines studied, indicating growth regulation by β_1-or β_2-ARs in these cells. The lowest concentration of propranolol, 50 nM, caused significant decrease in DNA synthesis only in the ER (+) cell lines, MCF-7 and MDA-MB-361, and in the ER (-) cell line MDA-MB-231. The decrease in DNA synthesis was concentration-dependent in ER (+) cell lines MCF-7 and ZR-75-1, and ER (-) cell line MDA-MB-453 at concentrations from 50 nM to 1 μM, suggesting that these cell lines may over-express β-AR. These finding suggest that binding of propranolol to these receptors likely did not saturate up to the 1 μM concentration. However, other cell lines studied exhibited an increase in DNA synthesis at 500 and 750 nM concentrations of propranolol, suggesting that propranolol may have caused a β-AR up-regulation in these cell lines or that these cell expressed excessive numbers of β-ARs.

Among the ER (+) cell lines, only MCF-7 responded to the β-1 specific antagonist atenolol. In this cell line, the inhibition in DNA synthesis was significant at concentrations from 250 nM to 1 μM. Lower concentrations were not effective. The ability of atenolol to significantly inhibit DNA synthesis in only MCF-7 suggests that

only this cell line may express β_1-ARs. The ER (-) cell line MDA-MB-468 was most responsive to increasing concentrations of atenolol from 250 nM to 1 μM. MDA-MB-453 cells were affected by atenolol treatment at both 750 nM and 1 μM concentrations while MDA-MB-231 were affected by atenolol only at the 750 nM concentration. The ER (-) cell line MDA-MB-435 did not respond to atenolol treatment, suggesting an absence of β_1-ARs. MDA-MB-468 cells were significantly more responsive to atenolol, suggesting the presence of up-regulated β_1-ARs.

The selective β_2-AR antagonist ICI 118,551 caused significant inhibitions in DNA synthesis in all cell lines tested. Both ER (+) and ER (-) breast cancer cell lines were affected by ICI 118,551 in a concentration-dependent manner, even though some of them exhibited somewhat different inhibition status at different concentrations. The most responsive cell line was the ER (+) cell line MDA-MB-361 in which DNA synthesis was inhibited even at the lowest concentration of ICI118,551 (50 nM). At 1 μM, the response reversed, suggesting possible receptor desensitization. The second ER (+) cell line MCF-7, also demonstrated a concentration-dependent inhibition of DNA synthesis by ICI 118,551 although to a lesser degree than MDA-MB-361 cells. Our results, at this point, implicate that these two ER (+) cell lines may express β_2-ARs. Earlier results from our laboratory have also shown that these two cell lines significantly responded to propranolol. The ER (+) cell line ZR-75-1 showed a significant reduction in DNA synthesis only at the highest concentration of ICI 118,551 (1 μM), indicating either the presence of constitutively over-expressed β_2-ARs or a lack of this receptor. However, the latter explanation seems unlikely in view of the significant response to

propranolol. Because we did not assess expression levels of β1/2-ARs in this project, these interpretations are speculative at this time.

All ER (-) cell lines responded to ICI 118,551 treatments. MDA-MB-453 cells showed an increasing decline in DNA synthesis with increasing concentrations of ICI 118,551. The response of this cell line to all β-AR antagonists, including the broad-spectrum β-AR antagonist propranolol, the β_1-AR antagonist atenolol and the β_2-AR antagonist ICI, 118, 551 suggests the presence of both, β_1-and β_2-ARs. However, in the other three ER (-) cell lines (MDA-MB-231, MDA-MB-435, and MDA-MB-468) DNA synthesis was inhibited up to 750 nM of ICI 118,551 while the effect reversed at the 1 μM concentration. This may reflect a desensitized β_2-AR in these cells even though at this concentration the inhibitions were still significant. Of particular interest was the finding that MDA-MB-231 cells responded to ICI 118,551 treatment most dramatically at each concentration up to 750 nM, indicating that this cell line may over-express β_2-ARs. Similarly, the ER (-) cell line MDA-MB-435 may also over-express or have only β_2-ARs because it responded significantly only to the broad-spectrum β-AR antagonist propranolol and to the β_2-AR antagonist ICI 118,551. Like MDA-MB-453 cells, ER (-) MDA-MB-468 cells were affected by all β-AR antagonists indicating expression of both, β_1-and β_2-ARs in these cells.

In summary, propranolol was more effective to inhibit DNA synthesis than either the selective β_2-antagonist ICI 118,551 or the selective β_1 antagonist atenolol. ER (+) cell lines generally were more responsive to the β_1-antagonist atenolol whereas ER (-) cell lines were more responsive to the β_2-antagonists ICI 118,551, suggesting that in ER

(+) cell lines β_1-ARs predominate while in ER (-) cell lines β_2-ARs predominate.

The β1/2-agonist isoproterenol significantly increased DNA synthesis only in ER (-) cell lines, with the exception of MDA-MB-468. The increases in DNA synthesis were concentration-dependent in MDA-MB-453 cells up to an almost two-fold increase at the 1 μM concentration of isoproterenol. This finding suggests the presence of β-AR in this cell line. In the ER (-) cell line MDA-MB-435, a maximum increase in DNA synthesis was already reached at the lowest concentration (50 nM) of isoproterenol. The extreme sensitivity of this cell line may be a reflection of over-expressed β-ARs. Interestingly, these two cell lines did not undergo any receptor desensitization at these concentrations, suggesting that the β-ARs responded to isoproterenol without intervening receptor dysregulation. The MDA-MB-231 cell line responded to isoproterenol treatment at only low concentrations and the increases in DNA synthesis started to decline at concentrations above 250 nM of isoproterenol. This may point to a down-regulation of β-ARs by high concentrations of isoproterenol. In addition, high concentrations of isoproterenol may nonselectively interact with other growth factors and their signaling pathways or activate genes that are growth inhibitory. On the other hand, the growth stimulatory effects of isoproterenol on the ER (-) cell lines may partly be explained by the fact that ER (-) breast cancer cell lines express many growth factors on their membranes and isoproterenol may interact or activate those growth factors, thus indirectly inducing cell proliferation.

The absence of any growth stimulatory effect of isoproterenol in MDA-MB-468 cells may partly be attributed to relatively low concentrations of isoproterenol used because this cell line responded very well to all β-AR antagonists. It also seems

possible that this cell line expressed constitutively activated β-ARs, which cannot be further stimulated by agonists but nevertheless respond to antagonists.

No ER (+) cell line responded significantly to isoproterenol treatment. There was no increase in DNA synthesis in MCF-7 cells in response to any isoproterenol concentration, while in the two other ER (+) cell lines (MDA-MB-361, ZR-75-1) DNA synthesis increased only slightly over basal levels. As these three ER (+) breast cancer cell lines significantly responded to all β-AR antagonists, they may have already been maximally stimulated by constitutively activated β-ARs. It is also possible that the relatively slow growth rate of ER (+) cell lines in comparison to ER (-) cell lines, may have required longer exposure times (several days) to yield detectable stimulatory effects.

The release of fatty acids from the plasma membrane by phospholipases might be increased by several factors, including, growth factors, adrenergic agonists, bradykinin, ACTH, serotonin, dopamine, angiotensin-II and musacarinic cholinergic agents (83). Even though fatty acids and specifically AA metabolism have been widely investigated for over 25 years, the exact mechanisms of action and effect of AA metabolism at the cellular level still remain to be uncovered. The release of AA from cellular membranes generally limits the synthesis of AA metabolites. AA is the precursor of a large family of compounds called eicosanoids, consisting of COX- derived PGs and LOX- derived LTs (22,23,71).

Because the controlled release of AA from the membranes by specific phospholipases not only is the first step in the production of several eicosanoids but also is an important aspect of cell signaling, alterations in AA release might be a crucial factor in many pathological conditions. The human breast cancer cell line BT-20 has been

shown to over-express EGF and TGF receptors, and the lipoxygenase inhibitor NDGA, inhibited DNA synthesis in this cell line (144). This suggests the possible involvement of lipoxygenase metabolites or genes modulated by LOX enzymes in tumor formation in this specific breast cancer line.

AA and its downstream products (eicosanoids) are considered crucial modulators of several cellular functions because eicosanoids are generally produced on demand by the rate limiting enzymes. Other than ion channels in which AA and eicosanoids have important impact, the G- proteins (GTP-binding proteins), a family of transducer molecules relaying signals from the membrane receptor-ligand interaction to the effector proteins regulating cellular responses, are also modulated by AA (83,145,146), suggesting that in addition to its direct action on the production of several mediators, AA may modulate several signaling pathways regulated by G-proteins. On the other hand, in platelets, the α subunits of G-proteins are arachidonoylated by post-translational modifications (146).

In adipose tissue, AA and other fatty acid metabolism are known to be regulated by c-AMP. The c-AMP increases PGE_2 production, activates $NF_{\kappa}B$ and causes COX-2 mRNA over-expression in macrophages (147). The n-6 polyunsaturated fatty acids increased adenylate cyclase in response to isoproterenol in pig adipocytes by enhancing the affinity of β-ARs for agonists (83). These findings suggest that the tumor promoting effects of n-6 dietary fats may partly mediated by an interaction with β-adrenergic regulatory pathways. In contrast, AA at concentrations of 100-300 μM decreased PKA activity in S49 lymphoma cells (83). This suggests that AA itself can be a protective factor against tumor progression unless it is metabolized to its downstream effectors.

This interpretation is supported by a number of studies, which have shown that exogenous or endogenous AA reduces tumor growth.

The enzyme $cPLA_2$ is involved in receptor activated signaling cascades and is phosphorylated by MAPK (22,148). Linoleic acid has a synergistic effect on the EGF-stimulated growth of mouse mammary epithelial cells in primary culture and it phosphorylated MAPK protein as well (30,149). The role of different fatty acids and particularly AA on tumor progression or initiation has also been attributed to the enhancer action of AA and free fatty acids (FFA) on PKC activation, because AA causes a sustained PKC activation in many tumor cells. PKC is rapidly and transiently activated by the diacylglycerol (DAG) and Inositol 1,4,5, triphosphate (IP_3) – mobilized intracellular Ca^{+2} derived from the membrane phospholipids (PIP_2) by the activity of PLC (83). PKC induces the MAPK cascade by activating RAF-1 kinase protein expression. PKC has also been implicated in the regulation of physiological and pathological processes, including cell proliferation, growth and differentiation.

Arachidonic acid and other free fatty acids (FFA) in the cytoplasm modulate several signal transduction pathway molecules, without undergoing metabolic conversion to the active eicosanoids (83). Several fatty acids, including AA, are considered as both modulator and second messenger. A modulator is a substance acting in a precise site for a very short time in a reversible manner to modify the properties and functions of a signal. Generally, the first messenger, a ligand or an agonist, depending on the cell type may act at the receptor level to modify several cellular activities. The second messenger, on the other hand, is generated in response to the first messenger and its concentration is altered by the primary signal and it may disappear as well as its action associates with the

physiological effects of the primary signal. The second messengers relay the first signal in a sequence of events acting either in the cytoplasm or nucleus in order to regulate different cellular functions and gene expressions. Fatty acid generation from the membrane phospholipids by different phospholipases and in response to c-AMP production from ATP by the AC enzyme are the examples of second messengers. Fatty acids are also modulators since they act in a reversible manner at specific intracellular sites for a short time to increase or decrease a physiological signal. Fatty acids modify the effects of several cellular functions, including protein kinases, G-proteins, adenylate and guanylate cyclases, phospholipases and almost all of the ion channels (82, 83,148,150).

The use of specific and general NSAIDs is being considered as a promising cancer prevention strategy, because of the documented protective effects of NSAIDs on cancer progression. In turn, this effect has been attributed to the inhibition of the PGs that have been implicated as cofactors in tumor formation and to the induction of cell cycle arrest and apoptosis (24,151,152) (Figure: 3). PGE2 production is controlled by two mechanisms: the regulation of substrate availability, mediated by PLA2 and the ability of cells to metabolize free AA to PGs, regulated by COX-2. The ER (-) breast cancer cell line, MDA-MB-231 expresses high levels of PLA_2 (153).

In this project, the FLAP inhibitor, MK886, was a very strong inhibitor of DNA synthesis in all breast cancer cell lines tested. MK886, a strong inhibitor of leukotriene synthesis, blocks the translocation of 5-LOX by changing the active site of FLAP (27). MK-886 was a very potent inhibitor of DNA synthesis for both ER (+) MCF-7 and ER (-) MDA-MB-453 cell lines. Even though the inhibition in DNA synthesis was

concentration-dependent in both cell lines, the ER (-) cell line MDA-MB-453 was much more responsive to MK-886 treatment. For the ER (+) MCF-7 breast cancer cell line, there was almost no difference in the inhibition of DNA synthesis at both 1 and 10 μM concentrations of MK-886. This may suggest a relative lack of LOX enzymes in the ER(+) cells, resulting in saturation of MK-886 binding already at low drug concentration.

Generally, high concentrations of NDGA inhibited DNA synthesis of both ER (+) and ER (-) cell lines. For the ER (-) cell lines, the first significant inhibition was observed at 5 μM concentration and continued up to 40 μM concentration. In the ER (-) MDA-MB-435 breast cancer cell line, the inhibition in DNA synthesis was considerably more pronounced than in other ER (-) cell lines. At a 40 μM NDGA concentration, there was almost complete inhibition of DNA synthesis this cell line. This may implicate a strong enzyme occupation by the inhibitor NDGA.

In summary, the reduction in DNA synthesis caused by the FLAP inhibitor MK-886 and by the non-specific LOX inhibitor NDGA was significantly higher in ER (-) cell lines than in ER (+) cell lines. This suggests that metastatic and invasive ER (-) breast cancer cells may over-express the lipoxygenase cascade. The strong inhibitory effect on DNA synthesis seen in the presence of both NDGA and MK-886 might be attributed to the inactivation of mitogenic signaling cascades or of genes driving cells to overgrow in the presence of LOX protein.

Generally, lower concentrations of NDGA and MK-886 than of the COX inhibitors were required to reduce DNA synthesis. Only the highest concentrations of the COX 1/2 inhibitor aspirin or the specific COX-2 inhibitor meloxicam did significantly reduce DNA synthesis in both ER (+) and ER (-) breast cancer cells. In the

ER (-) MDA-MB-453 cell line DNA synthesis was inhibited in a concentration-dependent manner at each concentration of aspirin, while in ER (+) MCF-7 cells the response reversed at the 1 μM concentration of aspirin. This difference may partly be attributed to higher COX-1/2 gene expression levels in the ER (-) breast cancer cell line. This interpretation is supported by reports that only ER (-) breast cancer cell lines over-express COX-2. Like aspirin, the COX-2 specific inhibitor meloxicam caused significant inhibitions in DNA synthesis of both ER (+) and ER (-) cell lines at only higher concentrations. The ER (+) ZR-75-1 and ER (-) MDA-MB-231 cell lines were significantly more responsive to meloxicam than the other cell lines.

Collectively, these results indicate that the COX pathway in these cell lines was either at a higher level of activity than the LOX pathway and therefore needed higher concentrations of inhibitors or that the cells have higher levels of COX protein than 5-LOX protein. On the other hand, the pharmacokinetic and pharmacodynamic properties of LOX pathway inhibitors might be more potent than COX inhibitors'. The cell proliferation results obtained in our study are in accord with two recent reports which showed that MDA-MB-231 breast cancer cells constitutively expressed the COX-2 gene accompanied by elevated PGE2 production (27,91), while MCF-7 and ZR-75-1 have been shown to express only COX-1 but not the COX-2 enzyme (27).

The fact that the 5-LOX inhibitor NDGA significantly inhibited cell proliferation in both ER (+) and ER (-) breast cancer cell lines whereas the selective COX-2 inhibitor meloxicam caused significant reduction only at higher concentrations suggests that the growth of these cells may be more dependent on leukotrienes than prostaglandins. On

the other hand, the lypoxygenase inhibitors may be more effective tumor suppressive compounds than the COX-2 inhibitors.

As yet unpublished data from our laboratory have shown that the ER (-) breast cancer cell line MDA-MB-435 expressed β2-AR as assessed by RT-PCR and that the β-AR agonist isoprotorenol, induced AA release in that cell line. Prospective studies will be conducted to determine whether or not β-AR –AA dependent growth regulating pathways modulate the expression of upstream or downstream effectors of both AA and other signaling cascades and induce transcription factor expression in breast cancer cells.

The expression of the COX-2 gene is rapidly increased by growth factors, tumor promoters and several cytokines and is markedly induced in a subset of cells including endothelial cells and colon, stomach, prostate, pancreatic and breast cancer cells (89,152,154-156). Lysophosphatidylcholine, one of the phospholipids on the cellular membranes, activated MAPK and transcription factors, CREB and ATF-1 with concomitant COX-2 over-expression in vascular endothelial cells, indicating that the activators of membrane phospholipids might induce COX-2 expression together with other cellular signal transduction elements to stimulate cell growth in many cells (157). In our study, high concentrations of both the COX 1/2 inhibitor aspirin and the COX-2 specific inhibitor meloxicam, inhibited DNA synthesis in all cell lines studied. Because in our study the COX 1/2 gene expression profile of the cell lines studied has not been determined, interpretation of this data is somewhat speculative.

There are a number of cross talks between different signaling cascades and that some NSAIDs inhibit cell growth and angiogenesis, while inducing apoptosis in a manner independent from their proposed activity. Therefore, it is imperative to take

into account the other actions of specific and general NSAIDs on cell regulation and control.

The ER (+) cell line MCF-7 and the ER (-) cell line MDA-MB-453 were treated with the K^{+1} channel inhibitor quinidine to asses the effects of potassium channels on DNA synthesis. Cell proliferation assays showed that quinidine significantly inhibited DNA synthesis in both cell lines. Quinidine was used at higher concentrations than β-AR agonist and antagonists based on effective concentration levels reported in the literature. The inhibition of DNA synthesis by quinidine was concentration-dependent in both cell lines. The ER (-) cell line MDA-MB-453 was more sensitive to quinidine than the ER (+) cell line MCF-7, as reflected by a significant response of MDA-MB-453 cells to the lowest concentration of quinidine (10 μM). On the other hand, the lowest concentration of quinidine to significantly reduce DNA synthesis in MCF-7 cell line was 20 μM.

Because quinidine is a blocker for all K^{+1} channels, including GIRK1, these data may indicate the involvement of several different potassium channel in the observed effect. The response to quinidine may be attributable to the over-expression of GIRK1 gene, because our RT-PCR studies indicate over-expression of GIRK1 in this cell line (Figure: 14). Accordingly, these finding suggest that in addition to their documented role the metastasis and invasion (130), the GIRKs may also directly regulate cell proliferation and growth in breast cancer cells. The use of novel GIRK1 inhibitors may thus be useful for the control of cellular growth and metastasis of breast tumors. The exact mechanism of action of GIRK1 in breast and other cancers still remains unclear and needs to be elucidated in the future.

Growth factors, hormones, receptors, inflammatory mediators and eicosanoids are considered to play crucial roles in the initiation and maintenance of tumor cell survival and growth. In response to the extracellular signals including hormones, growth factors, cytokines, antigens and endotoxins, the cells activate or inactivate specific transcription factors or genes in order to regulate diverse cellular processes such as cell proliferation, differentiation and growth and cellular metabolism. In order to understand the underlying mechanisms in the regulation of these cellular processes, the cellular signal transduction pathways modulating these events have to be elicited (158-161).

The role of signal transduction pathways in cancer formation and cell transformation is an established concept. In many epithelial cancers, the overexpressed or mutated receptors with their ligands bound by autocrine or paracrine loops to each other are the core factors in tumor formation. For example, EGF receptors regulate many pathways including Ras, PLC, Src tyrosine kinase, PI3K and protein tyrosine phosphatases. Ras, activated by EGF, regulates the MAPK (63,162,163). Signal transduction between MAPK in the cytoplasm and receptors on the cell membrane requires a series of events leading first to the formation of the activated Ras-Raf complex on the membrane, followed by initiation of the cytosolic MEK-MAPK pathway.

The MAPK pathway plays a significant role in the regulation of cellular processes including gene expression, cell survival, cell growth, apoptosis and cell motility. Components of the MAPK pathway have been shown to be mediators of phosphorylation of intracellular substrates including protein kinases, transcription factors and cell growth regulators (162). MAPK activity is mediated through three cascades including a MAPK, MAPK kinase (MAPKK, MKK, MEK) and a MAPKK kinase or MEK kinase

(MAPKKK or MEKK). The MAPK family includes at least four different subfamilies including extracellular signal-related kinases (ERK-1/2), Jun amino-terminal kinases (JNK1/2/3), p38 proteins (p38 α/β/δ/γ) that are regulated by distinct MAPKKs: MEK1/2 for ERK1/2, MKK3/6 for the p38, MKK4/7 (JNKK1/2) for the JNKs, and MEK 5 for ERK5 (158-160). Nevertheless, each of these specific MAPKK might be activated by more than one MAPKKK, leading to increases in the MAPK diversity (161).

The p38 MAPK and JNK pathways are induced by bacterial endotoxins, inflammatory cytokines and environmental stress, whereas ERKs are mainly activated by mitogens of the receptor tyrosine kinase family (163,164). These kinases are in turn induced by the upstream mitogen-activated protein / ERK kinases (MEKs) such as SAPK/ERK kinase-1 (SEK1, also known as MEK4), which is upstream of JNK/SAPK and MKK3/MKK6, which are upstream of p38 MAPK. These MEKs are in turn activated by MEK kinases, which MEKK 1, 2,3 and 5 have been, determined to date (165). Once activated, they can phosphorylate and activate a variety of transcription factors modulating several gene expressions. The dual phosphorylation of tyrosine (Y 185) and threonine (T 183) residues are required for the activation of the MAPK family proteins (8,116,117,185, 186). In the nucleus, most MAPKs phosphorylate Ets transcription factors (e.g., Elk-1, Cyclin D1, p21) that are involved in the activation of c-fos gene, whose products heterodimerize with the Jun proteins to form activator protein 1 (AP-1) complexes. The enhancing of AP-1 activity by MAPKs results in the cell proliferation by increasing cyclin D1 induction (159-160).

The inhibition of p38 MAPK by SC68376 inhibited cytokine-induced activation of COX-2 and PGE2 synthesis in NIH 3T3 cells. (164), suggesting that this pathway may

be a crucial signaling mechanism modulating cytokine- induced COX-2 gene expression and PG synthesis because inhibition of MAPK pathway may also inactivate or reduce the activation of PLA2. Furthermore, blocking of MEKK1-induced PGE2 production by a COX-2 inhibitor, NS-398, in NIH 3T3 cells indicating that COX- 2 is an important mediator in this process and that PLA2 is not the only target in MAPK pathway (164).

MAPK activation assays were performed for 6 and 24 hours because at shorter time periods no activation has been observed in response to isoproterenol. For MAPK studies, 1 μM isoproterenol has been used because it was the most effective concentration for cell proliferation studies. In MAPK (p44 / p42 ; ERK1 / ERK2) activation studies, our results show that in ER (+) MCF-7 cell line 1 μM isoproterenol slightly activated (phosphorylated) MAPK protein at 6 hours, but the activation ceased at 24 hours of exposure. In ER (-) MDA-MB-453 cell line, however, a slight activation in MAPK was seen at only 24 hours after 1 μM isoproterenol treatment. This may suggest that MAPK may be constitutively activated in these breast cancer cell lines and may not be affected by receptor-mediated activation.

The β1/2-AR antagonist 1 μM propranolol caused no significant change in the activation of MAPK protein in both ER (+) MCF-7 and ER (-) MDA-MB-453 breast cancer cell lines. The MEK, the upstream effector of MAPK, may be modulated by other growth factors or kinases so that the MAPK levels may not be changed in this cell line. Because MAPK serves a more common integration point among multiple cell signaling pathways, it is reasonable that MAPK can be less responsive to a single inhibitor, propranolol. In addition, another gene or another signaling pathway may be influencing the phosphorylation of MAPK in these cell lines, because phosphorylation of

the appropriate amino acid of the kinase protein is believed to modify the protein and expose the catalytic domain for subsequent substrate activation (158-160). Therefore, the discrepancies between the activation and expression of MAPK in response to propranolol treatment may partly be explained by a mechanism in which propranolol may not influence the direct phosphorylation of the MAPK during the same interval that it effects the MAPK expression.

In our studies on MAPK expression, 1 μM propranolol inhibited ERK1/2 expression in the ER (+) MCF-7 cell line at only 150 minutes of exposure while no significant inhibition in the expression status of MAPK was observed at earlier time intervals. In ER (-) MDA-MB-453 cells a reduction in the expression of MAPK was observed at both 60 and 150 minutes of exposure. The fact that propranolol did not cause inhibition in the MAPK expression up to 60 and 150 minutes may be attributed to a lack of effective receptor inhibition by propranolol or an over-abundance of β-ARs in these cells. In conjunction with the relatively weak inhibition of MAPK expression these findings also suggest that pathways other then the MAPK cascade were primarily affected by propranolol.

The specific β_1-AR antagonist atenolol (1 μM) did not change the expression of MAPK in ER (+) MCF-7 o ER (-) MDA-MB-453 breast cancer cells, suggesting that β_1-ARs did not signal via the MAPK cascade in these cells and that expression levels of MAPK protein may be dependent on additional growth factor pathways expressed in these cells.

The specific β_2-AR antagonist ICI 118,551 (1μM) significantly inhibited MAPK expression only in ER (-) MDA-MB-453 cells at 60 and 150 minutes of exposure.

There was no significant change in the levels of MAPK expression at other times. In the ER (+) cell line MCF-7, however, the expression levels of MAPK protein between treated and control cells remained unchanged after exposure to ICI 118,551. The responsiveness of MDA-MB-453 cells to this antagonist reflects the presence of β_2-AR in these cells and suggests that they signal via the MAPK cascade. On the other hand, the lack of responsiveness of MCF-7 cells may reflect an absence of β_2-ARs or constitutively high levels of expression of MAPK proteins.

The specific COX-2 inhibitor meloxicam was used at a concentration widely reported in the literature (100 μM), and cells were exposed to it for 24 hours to see its effects on MAPK expression. Meloxicam caused a slight decrease in total MAPK expression only in the ER (+) MCF-7 cells while it inhibited only ERK1 expression in ER (-) MDA-MB-231 cells. These findings suggests that meloxicam may utilize the MAPK cascade of both MCF-7 and MDA-MB-231 cells as a modulator. It also possible that meloxicam may reduce the production of several prostaglandins such as PGE2 in tumor cells. PGE2 is an activator of adenylate cyclase, which forms c-AMP from ATP. The PKA activated by c-AMP up-regulate the MAPK pathway (Figure 5 and 24). The inhibition of only one isoform of ERK (ERK1) by meloxicam in MDA-MB-231 cells may have been caused by ERK1 stabilization or high levels of preexisting ERK2 protein. Other cell lines, including the ER (-) MDA-MB-435 and MDA-MB-468 cell lines, and the ER (+) cell line ZR-75-1 did not exhibit differences in the expression status of MAPK in response to meloxicam. It is highly likely that these cells may be modulated by growth factors unrelated to the COX-2 enzyme. Interestingly, in one of the ER (-) cell lines (MDA-MB-453) meloxicam increased MAPK expression levels. This cell line

may have other growth factor receptors or genes that can be activated by COX-2 inhibitors, or COX-2 independent mechanism may be responsible for this effect.

The non-specific LOX inhibitor NDGA was used at 30 μM concentration in accordance with published procedures. In three ER (-) cell lines, MAPK expression was decreased by NDGA treatment. In the ER (-) cell lines MDA-MB-435 and MDA-MB-231, total MAPK expression was inhibited at 24 hours of exposure to NDGA, while in MDA-MB-468 cells, only the ERK1 isoform of MAPK was inhibited. These findings indicate that leukotrienes formed by LOX enzymes from AA may modulate the expression of MAPK in these cancer cells and is in accord with the often observed overexpression of LOX in breast cancer.

The ER (-) cell line MDA-MB-453 and two ER (+) cell lines (MCF-7 and ZR-75-1) did not respond to NDGA by changes in the expression of MAPK. This may reflect high base levels of MAPK protein expression that cannot be altered by NDGA or points to the influence of other non-NDGA -associated pathways effecting MAPK in these cells.

The changes in the expression of level MAPK protein by these inhibitors may partly be attributed to the increased levels of free AA in the cytoplasm. AA may inhibit the expression levels of MAPK, or reduced level of PGs and LTs may be responsible for the MAPK inhibition. On the other hand, when β-adrenergic antagonists are present, the decrease in PKA or c-AMP levels may directly or indirectly decrease the levels of MAPK protein.

Inhibition of the total (ERK1 and ERK2) MAPK protein expression levels by COX-2 inhibitors, 5-LOX inhibitors and β-adrenergic receptor antagonists without changes in the phosphorylation levels of ERK1 and ERK2 suggests that these inhibitors

are probably modulating the transcriptional expression of MAPK proteins with no effect either on the phosphorylation status of MAPK proteins or on the translation (mRNA) level of MAPK protein. This interpretation is in accord with reports that many post-translational modifications may occur at the protein level to decrease or modify the expected effect of any drug (159,161).

Overexpression of COX-2 during angiogenesis or neovascularization of many human epithelial cancers seems to be a characteristic feature of these cancers. The formation of new blood vessels by angiogenesis is a major requirement for the growth and metastatic spread of many tumors and NSAIDs have been shown to inhibit angiogenesis. COX-1 was necessary for the formation of endothelial tubes while COX-2 was required for the production of VEGF, one of the angiogenic factors (152), suggesting that in addition to their role in PG metabolisms, NSAIDs inhibiting both COX-1 and COX-2 may be crucial anti-angiogenic compounds that can prevent angiogenesis as well as metastasis. Masferrer et al. stated that mature blood vessels express COX-1, whereas newly formed cells express inducible COX-2, suggesting that several tumor-derived growth factors enhance angiogenesis by inducing the production of COX-2 –derived PGE2 (101,166). The levels of PGs synthesized by the COXs are increased in various cancers, including breast cancer and colon cancer. In addition, there are increased levels of PGE2 in the blood of cancer patients. (23,24,166).

PGE2 induces most of these effects through receptor-mediated elevation of c-AMP (116). Chemicals that inhibit PGE2 synthesis have been shown to block PGE2-induced c-AMP elevation in tumor cells (167). Because PGE2 has been shown to be secreted by both spontaneous and experimentally induced breast cancers and PGE2

receptors have been found in these cancer cells (167), the anti-proliferative effects of β_2-AR blockers observed in our studies may have been caused by the inhibition of both COX- 2 and PGE2 synthesis. In many cancer cell lines, blockers of lipoxygenase enzymes were more potent inhibitors of proliferation than blockers of cyclooxygenases.

Even though the mechanism by which COX-2 initiates tumor formation or causes growth and proliferation of tumor cells has not been exactly explained, DNA oxidation by COX-2 has been considered by some investigators as a crucial early step in tumor initiation since COX-2 reactions involve the formation of free oxygen radicals that may potentially damage cellular macromolecules, including DNA. During the production of PGG_2 from the AA, peroxyl radicals, which are a source of activated oxygen and might contribute to oxidation of other molecules, are produced and they may attack DNA causing mutation (168). Accordingly, COX-2-induced formation of reactive oxygen radicals were inhibited by both the NSAIDs and antioxidants, including phenol, ascorbic acid and dopamine (168,169). These findings suggest that over-activation of AA metabolism in the course of tumor induction may cause a genotoxic potential that promotes their progression to malignancy, because the free radicals COX-2 generates induces DNA mutations.

Receptors that initiate signal transduction pathways are activated by highly specific interactions with their specific ligands, leading to various biological responses including protein activation and gene expression. Nevertheless, transactivation or cross-talk between structurally unrelated receptors may occur and one receptor (GPCR or PG receptors) may modulate the activation or inhibition of another receptor (EGF) (Figure 24) (102,159).

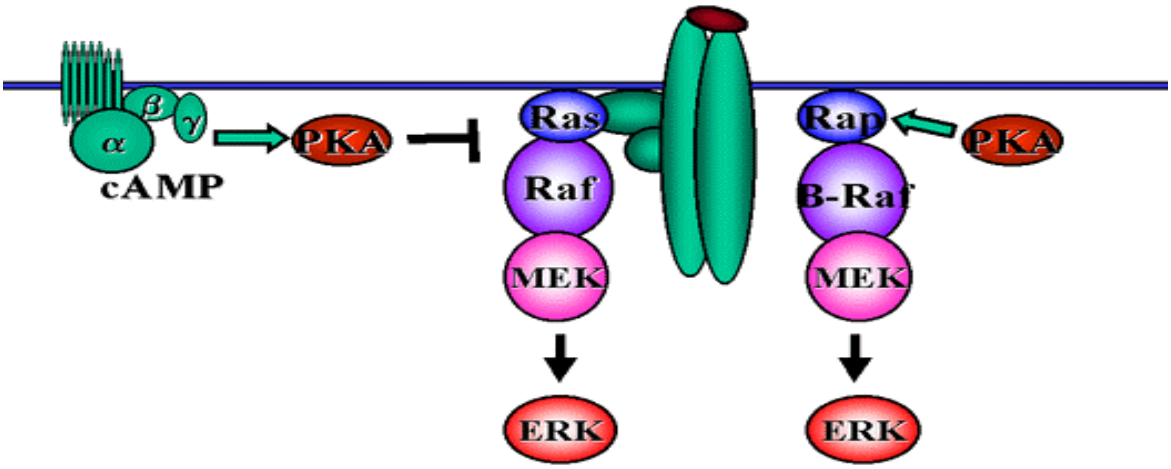


Figure 24: The cross-talk between signaling pathways. An example of how cross-talk structurally unrelated receptors may occur and how one receptor (GPCR) can modulate the activation or inhibition of another receptor (EGF).

Different signals may lead to an analogous response through similar components whereas stimulation of a cell with one specific ligand may cause more than one response. It has been shown that various extracellular stimuli, unrelated to the EGF-like ligands, may also induce the EGF receptor causing mitogenic responses (159). The inhibition of the MAPK pathway by beta-antagonists, 5-LOX and COX-2 antagonists observed by us may be partly explained by this kind of cross-talk among beta-ARs and the EGF and prostanoids receptors.

Unlike the RTKs (receptor tyrosine kinases), which include EGF, PDGF and insulin receptors with a catalytic activity to phosphorylate their targets, the GPCRs have no intrinsic enzymatic activity. Agonist activation of GPCRs leads to the induction of a wide variety of intracellular signaling cascades including modulation of adenylyl cyclases (AC), MAPKs, phospholipases, and ion channels (Figure: 5,6). GPCRs contain adrenergic, muscarinic, dopamine, adenosine, thrombin, serotonin, angiotensin II, extracellular Ca^{+2}, lysophosphatidic acid (LPA), yeast mating factor and Dictyostelium c-AMP receptors (102). Upon ligand binding, the GPCR undergoes a conformational change, which dramatically enhances its interaction with the G-protein (Figure 4). The heterotrimeric G-protein contains three kinds of subunits: α, β and γ. The α subunit having a GTPase activity binds to the G-nucleotide. The β/γ complex acts together as an inseparable unit enhancing receptor interaction with α subunits. Ligand binding to GPCR exchanges bound GDP for GTP resulting in the dissociation of the GTP-bound α subunit form β/γ subunit. The active GTP/α then regulates effector enzymes and ion channels. The β/γ complex also regulates effector proteins. The intrinsic GTPase activity of the α subunit ceases this signaling, and the α subunit then reunites with the β/γ

subunits so that the resting state is reached again (103). In the absence of activated receptors, the G-proteins stay predominantly in the inactive GDP state. The enzyme AC, which is mainly activated by the stimulatory α subunits ($G\alpha_s$), leads to the production of c-AMP (206). However, PLC-β is stimulated to various extents by the $G\alpha_q$ and Gβ/γ subunits and PKA can specifically inhibit Gβ/γ -induced PLC- β (66).

GPCRs can be desensitized by both uncoupling from their G-proteins and internalization. Long-term agonist-stimulated β2AR down-regulation contributes to the receptor desensitization and rapid inactivation causing receptor uncoupling. This process is initiated by phosphorylation of the agonist-occupied receptor (170). Desensitization effectively ceases the physiological effects of β-agonists in normal cells. Interestingly, GPCR endocytosis is needed for the β_2-adrenergic receptor-dependent induction of the MAPK pathway (171,172). Both receptors and G-proteins are the targets for the desensitization in which the different pathways have been well characterized. After a few minutes of incubation with the agonist, βARs are phosphorylated by PKA and GRKs (G-protein-coupled receptor kinases) or βARKs, leading to functional uncoupling of the receptor from the G-protein (110,171-175).

When receptor stimulation is constant for a longer time, receptor down-regulation contributes to the strengthening of desensitization through the degradation of pre-existing receptors and destabilization of receptor mRNA (104,110,113,170). The phosphorylation of β_2AR by GRK promotes the binding of β-arrestin to the receptor causing receptor internalization and blocking any further coupling to G_S (171, 172). Cancer cells that respond to long-term agonists stimulation, may loss their response to the

receptor desensitization and down-regulation and have distinct molecular characteristics and properties against stimulator and inhibitors resulting in aberrant cellular responses observed in many cancer cells. In other words, in these kinds of cancer cells, increased receptors recycling and decreased down-regulation of receptors may occur so that sustained activation of the receptors resulting in increased stimulation of downstream effectors may occur.

The β_2-Adrenergic receptors (β_2-ARs) are one of the G-protein coupled receptors mediating the effects of catecholamines and growth factors in a variety of cells. Many cell types are regulated by the distinct actions of β_2-ARs. The β_2-AR has an extracellular amino terminus, seven transmembrane spanning domains and an intracellular carboxyl terminus. The gene encoding β_2-AR has no intron and is located on chromosome 5q31-32 (105-108,113-115). Among the signaling pathways activated by the β_2-AR is the adenylyl cyclase/cAMP pathway (Figure: 4 and 6). c-AMP has been implicated in the modulation of a number of cellular functions including the control of cell growth and intracellular Ca^{+2} concentration. Depending on the type of cancer, cAMP may either stimulate or inhibit cell proliferation (61,66,105-107,109,142,176-182).

Cyclic AMP can modulate breast cancer proliferation through a variety of mechanisms. Cyclic AMP may regulate cell growth by binding to the regulatory subunits of the c-AMP dependent protein kinase (PKA). There are two main classes of PKA, PKAI (type I) and PKAII (type II), which have similar catalytic subunits but different regulatory subunits, termed RI and RII (183). Tumor specimens and some human cancer cell lines including breast cancer cell lines over-express only PKAI and / or its RI regulatory subunit, a phenomenon that has been related with tumor growth (184,185).

RI is also up-regulated during growth factor–mediated cell transformation (185). Furthermore, the c-AMP analog 8-Cl-cAMP, an RII site – specific analogue that down-regulates RI and suppresses the proliferation of several cancer cells, reduced cell growth in MCF-7 and MDA-MB-231 breast cancer cell lines as well as decreased the expression of the ras oncogene (180).

In contrast to the β-antagonists which inhibit cell growth, the β-AR agonist isoproterenol inhibited DNA synthesis of PC-3 prostate cancer cells and highly metastatic MDA-MB-231 breast cancer cells (186,187). Direct administration of membrane permeable c-AMP analogs resistant to degradation by phosphodiesterases also inhibited tumorigenesis of MCF-7 breast cancer cells (187), suggesting down-regulation of β_2-AR / cAMP resulting in a reversal of the agonist action observed in our studies. Furthermore, the actions of β-agonists in these cells in different laboratories may vary depending on variations in intracellular Ca^{+2} levels or K^{+1} channels, which have been implicated in intracellular signaling of β-ARs. Up-regulated PKA phosphorylates the β2AR resulting in a switch in the coupling of receptor from G_S to G_I (188), which might be responsible for the reported βAR inactivation and subsequent cell growth inhibition by β-AR agonists.

GIRK channels regulate the cell membrane potential as well as the frequency and duration of electrical impulses. Inhibition of GIRK channel activity may influence cardiac and brain function negatively. The G protein-linked receptor subtypes that activate these channels include muscarinic, GABA, serotonin (5HT1A), adenosine (P1), somatostatin, enkephalin, α_2-adrenergic, β_2-adrenergic and dopamine (D2) receptors (117). GIRK1 is activated by a variety of hormone and neurotransmitters including

epinephrine, norepinephrine, 5-HT, dopamine, GABA, adenosine, opioids, acetylcholine and somatostatin (120).

We selected the ER (-) cell line MDA-MB-453 to investigate the expression status of GIRK1 in control and propranolol-exposed cells by relative competitive RT-PCR. GIRK1 was over-expressed when the cells were exposed continuously to propranolol for 7 days. However, expression levels of GIRK1 remained unchanged when the cells were treated with propranolol for 30 minutes every day for a 7-day period. The over-expression of GIRK1 observed after continuous exposure to propranolol may be attributable to up-regulation of β-ARs in response to chronic exposure to the β-AR-antagonist. In one study, GIRK1 was over-expressed in breast tumor tissues associated with lymph node metastases. GIRK1 was not expressed in the adjacent normal breast tissue. It was also shown that GIRK1 was over-expressed in the corresponding lymph node only if the primary tumor over-expressed GIRK1 (130). This study suggests that GIRK1 might be a crucial marker for the identification of highly aggressive, metastatic breast cancer and may play a role in the growth regulation of these cancers that may be targeted for cancer preventive and therapeutic strategies.

The GIRK channel has a conserved peptide sequence, arginine-glycine-aspartate (RGD), to which another class of membrane proteins, integrins, may bind. The mutation of this recognition site on the channel blocks this binding, and without this binding, the GIRK current is reduced or stopped (189). Because integrins have a wide variety of functions, including cell adhesion, migration, survival, growth, proliferation, differentiation, and modulation of gene expression, the integrin-GIRK interaction might be important in cell migration and signal transduction pathways. Integrin signaling has

been associated with gene activation, src tyrosine kinases, MAPKs and focal adhesion kinases. The overexpression of GIRK1 in the invasive breast cancers may partly be explained by this GIRK – integrin interactions due to the fact that integrins are needed for cancer cells to metastasize and migrate to the distant organs. In fact, in neuroblastoma cell lines, the cell migration and neurite formation have been associated with integrin, with the activation of a GIRK channel, and with tyrosine phosphorylation (189). With respect to our studies, over-expression of GIRK may also be associated with modulations in β-ARs and their associated signal transduction pathways.

CHAPTER 4: CONCLUSION / SUMMARY

The identification of crucial sites and detailed knowledge of receptor-activated down-stream events in growth-regulating signal transduction pathways of breast cancer cells provides a basis for the development of novel therapeutic strategies by targeting specific components of a given signal transduction pathway. Understanding the biochemistry and physiology of a pathway or a specific gene important for cancer progression or prevention will provide many opportunities for enhanced clinical management of cancer patients who are at high risk because they have mutations in these genes or proteins that regulate several crucial pathways. Modern drug development strategies thus involve the identification of enzymatic steps that can be inhibited by a pharmaceutical agent, protein-protein interactions that may be changed, or protein-signaling molecule interactions that may be appropriately mimicked or suppressed by a drug. This approach may allow to block tumor cell proliferation, inhibition of gene or protein expression, stimulation of apoptosis, inactivation of the cell cycle, as well as inhibition of angiogenesis and metastasis.

Data generated by this study suggest that β-AR, K^{+1} channel, COX-1/2, FLAP and 5-LOX antagonists,- dependent on the cell type and concentrations-, decrease cell proliferation in vitro in both ER (-) and (+) breast cancer cell lines, indicating that these agents may be valuable tools for the control or inhibition of breast cancer growth. Among the β-antagonists, propranolol was more effective than the selective β_2-antagonist

ICI 118,551 or the β_1-antagonist atenolol. ER (+) cell lines were generally more responsive to the β_1-antagonist atenolol, whereas ER (-) cell lines were more responsive to the β_2-antagonists ICI 118,551. These findings suggests that in the ER (+) cell lines β1-ARs predominate while in the ER (-) cell lines the majority of β-ARs were of the β_2-tye. The reduction in DNA synthesis caused by the FLAP inhibitor MK-886 and the 5-LOX inhibitor NDGA was significantly higher in ER (-) cell lines than in the ER (+) cell lines. On the other hand, the β-agonist isoproterenol significantly increased DNA synthesis only in ER (-) cell lines, suggesting that in the non-responsive cell lines β-ARs may have been constitutively activated resulting in maximum growth stimulation without the addition of external stimuli.

The observed ability of COX inhibitors, 5-LOX inhibitors, β-adrenergic blockers and K^+ channel blockers to suppress the growth and proliferation of the a subset of breast cancer cells lines is an important finding that provides the basis for the exploration of such agents in the clinical management and prevention of breast cancer. Because these inhibitors are already widely used for the treatment of various diseases they can immediately be used in clinical trials with breast cancer patients. In particular the β-ARs represent a promising new target for the development of new drug candidates with potential application in the clinical fields because a wealth of information is already available on the biochemical and molecular events underlying signaling by these receptors.

REFERENCES

REFERENCES

1. Lipworth, L., Epidemiology of breast cancer, Eur J Cancer Prev, 4 (1995), 7-30.

2. Greenlee, RT., Hill-Harmon, MB., Murray, T., Thun, M., Cancer Statistics, CA Cancer J Clin, 51 (2001), 15-36.

3. Alberg AJ., and Helzlsouer, KJ., Epidemiology, prevention, and early detection of breast cancer, Curr Opin Oncol, 9 (1997), 505-11.

4. Freudenheim, JL., Marshall JR., Vena, JE., Moysich, KB., Muti, P., Laughlin, R., Nemoto, T., Graham, S., Lactation history and breast cancer risk, Am J Epidemiol, 146 (1997), 932-8.

5. Ellisen, LW., Haber, DA., Hereditary breast cancer, Annu. Rev. Medicine, 49 (1998) 425-36.

6. Buchholz, TA., Weil, MW., Story, MD., Strom, EA., Brock, WA., McNeese, MD., Tumor suppressor genes and breast cancer, Rad Oncol Invest, 7 (1999), 55-65.

7. Ingvarsson, S., Molecular genetics of breast cancer progression, Seminars in Cancer Biology 9 (1999), 277-288.

8. Antoniou, AC., Pharoah, PD., McMullan, G., Day, NE., Ponder BA, Easton, D., Evidence for further breast cancer susceptibility genes in addition to BRCA1 and BRCA2 in a population-based study, Genet Epidemiol 21(2001), 1-18.

9. Grodstein, F., Stampfer, MJ., Colditz, GA., et al., Postmenopausal hormone therapy and mortality, New England Journal of Medicine, 336 (1997) 1769-1775.

10. Roy, JA., Sawka, CA., Pritchard, KI., Hormone replacement therapy in women with breast cancer: do the risks outweigh the benefits?, Journal of Clinical Oncology, 14 (1996) 997-1006.

11. Newman, B., Millikan, RC., King, MC., Genetic epidemiology of breast and ovarian cancers, Epidemiol Rev, 19 (1997), 69-79.

12. Schuller, HM., Cell type specific, receptor-mediated modulation of growth kinetics in human lung cancer cell lines by nicotine and tobacco-related nitrosamines, Biochem Pharmacol, 38 (1989), 3439-42.

13. Schuller, HM., Orloff, M., Tobacco-specific carcinogenic nitrosamines. Ligands for nicotinic acetylcholine receptors in human lung cancer cells, Biochem Pharmacol, 55 (1998), 1377-84.

14. Schuller, HM., Carbon dioxide potentiates the mitogenic effects of nicotine and its carcinogenic derivative, NNK, in normal and neoplastic neuroendocrine lung cells via stimulation of autocrine and protein kinase C-dependent mitogenic pathways, Neurotoxicology, 15(1994), 877-886.

15. Schuller, HM., Tithof, PK., Williams, M., Plummer, H 3rd., The tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone is a beta-adrenergic agonist and stimulates DNA synthesis in lung adenocarcinoma via beta-adrenergic receptor-mediated release of arachidonic acid, Cancer Res,59 (1999), 4510-5.

16. Banerjee, SP., Sharma, VK et al., Alterations in beta-adrenergic receptor binding during ethanol withdrawal, Nature, 276 (1978), 407.

17. Driver, HE., Swann, P., Alcohol and human cancer, Anticancer Research, 7 (1987), 309.

18. Koga, Y., Sufu, M., et al. Alterations in beta–adrenergic receptor density and c-AMP levels in the myocardium of rats chronically treated with alcohol, The Kurume Med Journal, 40 (1993), 1-18.

19. Taketo, MM., Cyclooxygenase-2 inhibitors in tumorigenesis (Part I), J Natl Cancer Inst, 90 (1998), 1529-1536.

20. Taketo, MM. Cyclooxygenase-2 inhibitors in tumorigenesis (Part II), J Natl Cancer Inst, 90 (1998), 1609-1620.

21. Paine, E., Palmantier, R., Akiyama, SK., Olden, K., Roberts, JD., Arachidonic acid activates mitogen-activated protein (MAP) kinase-activated protein kinase 2 and mediates adhesion of a human breast carcinoma cell line to collagen type IV through a p38 MAP kinase-dependent pathway, J Biol Chem, 275 (2000), 11284-11290.

22. Leslie, CC., Properties and Regulation of Cytosolic Phospholipase A_2, J. Biol. Chem, 272 (1997), 16709-16712.

23. Ara, G., Teicher, BA., Cyclooxygenase and lipoxygenase inhibitors in cancer therapy, Prostaglandins Leukot Essent Fatty Acids, 54 (1996), 3-16.

24. Dubois, RN., Abramson, SB., Crofford, L., Gupta, RA., Simon, LS, Van De Putte LB, Lipsky, PE., Cyclooxygenase in biology and disease, FASEB J 12 (1998), 1063-73.

25. Thompson, HJ., Jiang, C., Lu, JX., Mehta, RG., Piazza, GA., Paranka, NS., Pamukcu, R., and Ahnen, DJ., Sulfone metabolite of sulindac inhibits mammary carcinogenesis, Cancer Res. **57** (1997), 267–271.

26. Lim, JW., Kim, H., Kim, KH., Nuclear Factor $_{\kappa}$B Regulates COX-2 Expression and Cell Proliferation in Human Gastric Cancer Cells, Lab. Invest, 81 (2001), 349-360.

27. Hong, SH., Avis, I., Vos, MD., Martínez, A., Treston, AM., Mulshine, JL., Relationship of Arachidonic Acid Metabolizing Enzyme Expression in Epithelial Cancer Cell Lines to the Growth Effect of Selective Biochemical Inhibitors, Cancer Res, 59 (1999), 2223-2228.

28. Palmantier, R., Roberts, JD., Glasgow, WC., Eling, T., Olden, K., Regulation of the adhesion of a human breast carcinoma cell line to type IV collagen and vitronectin: roles for lipoxygenase and protein kinase C, Cancer Res, 56 (1996), 2206-12.

29. Burstein, S, Gagnon G, Hunter SA, Maudsley DV., Elevation of prostaglandin and cyclic AMP levels by arachidonic acid in primary epithelial cell cultures of C3H mouse mammary tumors, Prostaglandins, 13 (1977), 41-53.

30. Rose, DP., Connolly, JM., Effects of fatty acids and inhibitors of eicosanoid synthesis on the growth of a human breast cancer cell line in culture, Cancer Res, 50 (1990), 7139-44.

31. Noguchi, M., Rose, DP., Earashi, M., Miyazaki, I., The role of fatty acids and eicosanoid synthesis inhibitors in breast carcinoma, Oncology, 52(1995), 265-71.

32. Noguchi, M., Earashi, M., Miyazaki, I., Tanaka, M., Sasaki, T., Effects of indomethacin with or without linoleic acid on human breast cancer cells in vitro. Prostaglandins Leukot Essent Fatty Acids, 52 (1995), 381-6.

33. Connolly, JM., Liu, XH., Rose, DP., Dietary linoleic acid-stimulated human breast cancer cell growth and metastasis in nude mice and their suppression by indomethacin, a cyclooxygenase inhibitor, Nutr Cancer, 25(1996), 231-40.

34. Bartsch, H., Nair, J., Owen, RW., Dietary polyunsaturated fatty acids and cancers of the breast and colorectum: emerging evidence for their role as risk modifiers, Carcinogenesis, 20(1999), 2209-18.

35. Rose, DP., Dietary fatty acids and cancer, Am J Clin Nutr, 66 (1997), 998S-1003S.

36. Rose, DP., Effects of dietary fatty acids on breast and prostate cancers: evidence from in vitro experiments and animal studies, Am J Clin Nutr, 66 (1997), 1513S-1522S.

37. Henderson, BE., and Feigelson, HS., Hormonal carcinogenesis, Carcinogenesis, 21 (2000), 427-433.

38. Thomas, HV., Key, TJ., Allen, DS., Moore, JW., Dowsett, M., Fentiman, IS., Wang, DY., A prospective study of endogenous serum hormone concentrations and breast cancer risk in premenopausal women on the island of Guernsey, Br J Cancer, 75 (1997), 1075-9.

39. Khan, SA., Rogers, MA., Khurana, KK., Meguid, MM., Numann, PJ., Estrogen receptor expression in benign breast epithelium and breast cancer risk. J Natl Cancer Inst, 90 (1998), 37-42.

40. Khan, SA., Rogers, MA., Obando, JA., Tamsen, A**.,** Estrogen receptor expression of benign breast epithelium and its association with breast cancer. Cancer Res, 54 (1994), 993-7.

41. van Agthoven, T., Timmermans, M., Foekens, JA., Dorssers, LC., Henzen-Logmans, SC., Differential expression of estrogen, progesterone, and epidermal growth factor receptors in normal, benign, and malignant human breast tissues using dual staining immunohistochemistry, Am J Pathol, 144(1994), 1238-46.

42. Clemons, M., Goss, P., Estrogen and the risk of breast cancer, New Eng J Med, 344 (2001), 276-285.

43. Barrett-Lee, PJ., Travers, MT., McClelland, RA., Luqmani, Y., Coombes, RC., Characterization of estrogen receptor messenger RNA in human breast cancer, Cancer Res, 47 (1987), 6653-9.

44. Kumar, V., Green, S., Stack, G., Berry, M., Jin, JR., Chambon, P., Functional domains of the human estrogen receptor, Cell, 51 (1987), 941-51.

45. Yager, JD., Liehr, JG., Molecular mechanisms of estrogen carcinogenesis, Annu Rev Pharmacol Toxicol, 36 (1996), 203-32.

46. Cavalieri, EL., Stack, DE., Devanesan, PD., Todorovic, R., Dwivedy I, Higginbotham, S., Johansson SL, Patil KD, Gross, ML., Gooden JK, Ramanathan, R., Cerny, RL., Rogan, EG., Molecular origin of cancer: catechol estrogen-3,4-quinones as endogenous tumor initiators, Proc Natl Acad Sci U S A, 94(1997),10937-42.

47. Zhu, BT., Conney, AH., Functional role of estrogen metabolism in target cells: review and perspectives, Carcinogenesis, 19 (1998), 1-27.

48. Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E., Auricchio, F., Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells, EMBO J, 15 (1996),1292-300.

49. Improta-Brears, T., Whorton, AR., Codazzi F, York, JD, Meyer, T., McDonnell, DP., Estrogen-induced activation of mitogen-activated protein kinase requires mobilization of intracellular calcium, Proc Natl Acad Sci U S A, 96 (1999), 4686-91.

50. Endoh, H., Sasaki, H., Maruyama, K., Takeyama, K., Waga I, Shimizu T, Kato S, Kawashima, H**.,** Rapid activation of MAP kinase by estrogen in the bone cell line, Biochem Biophys Res Commun, 235(1997), 99-102.

51. Benten, WP., Stephan, C., Lieberherr, M., Wunderlich, F., Estradiol signaling via sequestrable surface receptors, Endocrinology, 142(2001), 1669-77.

52. Levin, ER., Cellular Functions of the Plasma Membrane Estrogen Receptor, Trends Endocrinol Metab, 10 (1999),374-377.

53. Bates, SE., Valverius, EM., Ennis, BW., Bronzert, DA., Sheridan, JP., Stampfer MR, Mendelsohn, J, Lippman, ME., Dickson, RB., Expression of the transforming growth factor-alpha/epidermal growth factor receptor pathway in normal human breast epithelial cells, Endocrinology, 126 (1990), 596-607.

54. Walker, RA., and Dearing, SJ., Expression of epidermal growth factor receptor mRNA and protein in primary breast carcinomas, Breast Cancer Res Treat, 53(1999), 167-76.

55. Nicholson, RI., McClelland, RA., Gee, JM., Manning, DL., Cannon, P., Robertson, JF., Ellis, IO., Blamey, RW., Epidermal growth factor receptor expression in breast cancer: association with response to endocrine therapy, Breast Cancer Res Treat, 29(1994),117-25.

56. Biscardi, JS., Tice DA, Parsons, SJ., c-Src, receptor tyrosine kinases, and human cancer, Adv Cancer Res, 76 (1999), 61-119.

57. Biscardi, JS., Ishizawar, RC., Silva CM, Parsons, SJ., Tyrosine kinase signalling in breast cancer: epidermal growth factor receptor and c-Src interactions in breast cancer, Breast Cancer Res, 2(2000), 203-10.

58. DeFazio, A., Chiew, YE., McEvoy, M., Watts, CK., Sutherland, RL., Antisense estrogen receptor RNA expression increases epidermal growth factor receptor gene expression in breast cancer cells, Cell Growth Differ, 8(1997), 903-11.

59. Cho, H., and Katzenellenbogen, BS., Synergistic activation of estrogen receptor-mediated transcription by estradiol and protein kinase activators, Mol Endocrinol, (1993),441-52.

60. Ince, BA., Montano, MM., Katzenellenbogen, BS., Activation of transcriptionally inactive human estrogen receptors by cyclic adenosine 3',5'-monophosphate and ligands including antiestrogens, Mol Endocrinol, 1994 (1994), 1397-406.

61. Aronica, SM., Kraus WL, Katzenellenbogen, BS., Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription, Proc Natl Acad Sci U S A. 1994 Aug 30;91(18):8517-21.

62. Fujimoto,N., and Katzenellenbogen, BS., Alteration in the agonist/antagonist balance of antiestrogens by activation of protein kinase A signaling pathways in breast cancer cells: antiestrogen selectivity and promoter dependence Mol. Endocrinol, 8 (1994) : 296-304.

63. Cano E, Mahadevan LC. Parallel signal processing among mammalian MAPKs, Trends Biochem Sci 20 (1995),117-22.

64. Chen, D., Pace, PE., Ali, S., Phosphorylation of Human Estrogen Receptor [alpha] by Protein Kinase A Regulates Dimerization, Mol Cell Biol, 19 (1999), 1002-1015

65. Le Goff, P., Montano, MM., Schodin, DJ., Katzenellenbogen, BS., Phosphorylation of the human estrogen receptor. Identification of hormone-regulated sites and examination of their influence on transcriptional activity, J Biol Chem. 269(1994), 4458-66.

66. Guilbaud, NF., Gas, N., Dupont, MA., Valette, A., Effects of differentiation-inducing agents on maturation of human MCF-7 breast cancer cells, J Cell Physiol, 145(1990):162-72.

67. Hussey, TJ., and Tisdale, M., Inhibition of tumor growth by lipoxygenase inhibitors, Br. J. Cancer, 74 (1996), 683-687.

68. Hong, SH., Avis, I., Vos MD, Martinez A, Treston, AM., Mulshine, JL., Relationship of arachidonic acid metabolizing enzyme expression in epithelial cancer cell lines to the growth effect of selective biochemical inhibitors, Cancer Res, 59(1999):2223-8.

69. Ghosh, J., and Myers CE., Arachidonic acid stimulates prostate cancer cell growth: critical role of 5-LOX. Biochem. Biophys. Res. Commun, 235 (1997), 418-423.

70. Avis, IM, Jett M, Boyle T, Vos MD, Moody, T, Treston, AM., Martinez, A., Mulshine, JL., Growth control of lung cancer by interruption of 5-lipoxygenase-mediated growth factor signaling, J Clin Invest, 97(1996), 806-13.

71. Lin, LL., Wartmann, M., Lin, AY., Knopf, JL, Seth A, Davis, RJ., cPLA2 is phosphorylated and activated by MAP kinase. Cell, 72 (1993), 269-78.

72. Schievella, AR., Regier, MK., Smith, WL., Lin, LL., Calcium-mediated translocation of cytosolic phospholipase A2 to the nuclear envelope and endoplasmic reticulum, J Biol Chem, 270(1995), 30749-54.

73. Brock, TG., Robert W. McNish, and Marc Peters-Golden. Arachidonic Acid Is Preferentially Metabolized by Cyclooxygenase-2 to Prostacyclin and Prostaglandin E2. J. Biol. Chem, (1999) 274: 11660-11666.

74. Yip-Schneider, MT., Barnard, DS., Billings, SD., Cheng, L., Heilman, DK, Sweeney CJ., Cyclooxygenase-2 expression in human pancreatic adenocarcinomas: Carcinogenesis 21(2000),139-46.

75. Heasley, LE., Thaler, S., Nicks, M., Price, B., Skorecki, K., Nemenoff, RA., Induction of cytosolic phospholipase A2 by oncogenic Ras in human non-small cell lung cancer, J Biol Chem, 272(1997),14501-4.

76. Van Putten, V., Refaat, Z., Dessev, C., Blaine, S., Wick, M., Butterfield, L., Han, SY., Heasley, LE, Nemenoff, RA., Induction of cytosolic phospholipase A2 by oncogenic Ras is mediated through the JNK and ERK pathways in rat epithelial cells, J Biol Chem. 276(2001), 1226-32.

77. Robbins, DJ., Zhen, E., Owaki, H., Vanderbilt, CA., Ebert D, Geppert TD, Cobb MH., Regulation and properties of extracellular signal-regulated protein kinases 1 and 2 in vitro, J Biol Chem, 268(1993),5097-106.

78. Chen, RH., and Blenis, J., Nuclear localization and regulation of erk- and rsk-encoded protein kinases, Mol. Cell. Biol, 12 (1992): 915-927.

79. Lenormand, P., Sardet, C., Pages, G., L'Allemain, G., Brunet, A., Pouyssegur, J., Growth factors induce nuclear translocation of MAP kinases (p42mapk and p44mapk) but not of their activator MAP kinase kinase (p45mapkk) in fibroblasts, J Cell Biol. 1993 122(5): 1079-88.

80. Piomelli, D., Arachidonic acid in cell signaling. Curr Opin Cell Biol 5 (1993), 274-83.

81. Harrison KA, Murphy RC., Isoleukotrienes are biologically active free radical products of lipid peroxidation, J Biol Chem, 270 (1995),17273-8.

82. Meves, H. Modulation of ion channels by arachidonic acid, Prog. Neurobiol, 43 (1994), 175-186.

83. Graber, R., Sumida, C., Nunez, EA., Fatty acids and cell signal transduction, J. Lipid Mediators Cell Signalling, 9 (1994), 91-116.

84. Brueggemeier, RW., Quinn, AL., Parrett, ML., Joarder, FS., Harris, RE., Robertson FM., Correlation of aromatase and cyclooxygenase gene expression in human breast cancer specimens. Cancer Lett, 140(1999):27-35.

85. Lee, PP., and Ip, MM., Regulation of proliferation of rat mammary tumor cells by inhibitors of cyclooxygenase and lipoxygenase. Prostaglandins Leukot Essent Fatty Acids. (1992), 21-31.

86. Smith, W.L., Garavito, R.M., and DeWitt, DL., Prostaglandin Endoperoxide H Synthases (Cyclooxygenases)-1 and –2, J. Biol Chem, 271 (1996), 33157-33160.

87. Harris, RE., Namboodiri, KK., Farrar, WB., Nonsteroidal antiinflammatory drugs and breast cancer, Epidemiology, 7 (1996), 203-205.

88. Prescott, SM., Fitzpatric, FA., Cyclooxyenase-2 and carcinogenesis, Biochim. Biopys Acta, 1470 (2000), M69-M78.

89. Subbaramaiah, K., Telang, N., Araki, B., DeVito, BB., Dannenberg, AJ., Transcription of cyclooxygenase-2 is enhanced in transformed mammary epithelial cells, Cancer Res, 56 (1996), 4424-4429.

90. Slice, LW., Bui, L., Mak, C., Walsh, JH., Differential regulation of COX-2 transcription by Ras- and Rho-family of GTPases, Biochem Biophys Res Commun. 276(2000), 406-10.

91. Liu, XH., and Rose, DP., Differential expression and regulation of cyclooxygenase-1 and -2 in two human breast cancer cell lines, Cancer Res, 56 (1996), 5125-5127.

92. Gilhooly, EM, Rose DP., The association between a mutated ras gene and cyclooxygenase-2 expression in human breast cancer cell lines. Int J Oncol. (1999), 267-70.

93. Tjandrawinata, RR., Dahiya, R., Hughes-Fulford, M., Induction of cyclooxygenase-2 mRNA by PGE2 in human prostatic carcinoma cells, Bri. J. Cancer 75 (1997), 1111-1118.

94. Sovak, MA., Bellas, RE., Kim DW., Zanieski, GJ, Rogers, AE., Traish, AM., Sonenshein, GE., Aberrant $NF_{\kappa}B$/Rel expression in the pathogenesis of breast cancer, J Clin Invest, 100 (1997), 2952-2960.

95. David J. Wadleigh, Srinivasa T. Reddy, Elizabeth Kopp, Sankar Ghosh, and Harvey R. Herschman, Transcriptional Activation of the Cyclooxygenase-2 Gene in Endotoxin-treated RAW 264.7 Macrophages, J. Biol. Chem, 275 (2000): 6259-6266.

96. Tsujii, M., and DuBois, RN., Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase-2, Cell 83 (1995), 493–501.

97. Zhang, X., Morham, SC., Langenbach, R., and Young, D.. Malignant transformation and antineoplastic actions of NSAIDs on cyclooxygenase-null embryo fibroblasts. J Exp Med, 190 (1999), 451-459.\

98. Luchian, T., Schreibmayer, W., Ion permeation through a G-protein activated (GIRK1/GIRK5) inwardly rectifying potassium channel, Biochim Biophys Acta, 1368 (1998),167-70.

99. Inoue, H., Nanayama, T., Hara, S., Yokoyama, C., Tanabe, T., The cyclic AMP response element plays an essential role in the expression of the human prostaglandin-endoperoxide synthase 2 gene in differentiated U937 monocytic cells, FEBS Lett, 350(1994),51-4.

100. Oshima, T., Yoshimoto, T., Yamamoto, S., Tanabec, T., c-AMP-dependent induction of fatty acid cyclooxygenase mRNA in mouse osteoblastic cells (MC3T3-E1), J. Biol. Chem, 266 (1991), 13621-13626.

101. Prescott, SM., Fitzpatric, FA. (2000). Cyclooxygebnase-2 and carcinogenesis. Biochim. Biopys Acta. 1470: M69-M78.

102. Schenk, PW., Snaar-Jagalska, BE., Signal perception and transduction: the role of protein kinases, Biochim Biophys Acta, 1449 (1999), 1-24.

103. Hamm, HE., The many faces of G protein signaling, J. Biol. Chem, 273 (1998), 669-78.

104. Ji, TH., Grossman, M., Ji, I., G-protein-coupled receptors, JBC, 273 (1998): 17299-05.

105. Osborne, KC., Steroid hormone receptors in breast cancer management. Breast Cancer Research and Treatment. 51 (1998), 227-138.

106. Marchetti, B., Spinola, PG., Plante M, Poyet P, Follea N, Pelletier G, Labrie F., Beta-adrenergic receptors in DMBA-induced rat mammary tumors: correlation with progesterone receptor and tumor growth, Breast Cancer Res Treat, 13(1989), 251-63.

107. Marchetti, B., Spinola, PG., Pelletier, G., Labrie, F,. A potential role for catecholamines in the development and progression of carcinogen-induced mammary tumors: hormonal control of beta-adrenergic receptors and correlation with tumor growth, J Steroid Biochem Mol Biol, 38(1991). 307-20.

108. Ruffolo, RR., et al. Alpha and beta-adrenoceptors: from gene to the clinic: Structure -activity relationship and therapeutic applications. Journal of Medical Chemistry, 38 (1994), 3682.

109. Walsh, DA., and Van Patten SM., Multiple pathway signal transduction by the cAMP-dependent protein kinase, FASEB J, 8 (1994),227-36.

110. Jockers, R., Angers, S., Da Silva, A., Benaroch, P., Strosberg, AD., Bouvier, M., Marullo, S., Beta(2)-adrenergic receptor down-regulation. Evidence for a pathway that does not require endocytosis, J Biol Chem, 274 (1999), 28900-8.

111. Daniel, PB., Cyclic AMP signaling and gene regulation, Annu. Rev. Nutr, 18 (1998), 353-383.

112. Montminy, M., Transcriptional regulation by c-AMP, Annu. Rev. Biochem, 66 (1997), 807-822.

113. Sprang, SR., G protein mechanisms: insights from structural analysis. Annu. Rev. Biochem, 66 (1997), 639-59.

114. Crespo, P., Cachero TG, Xu N, Gutkind, JS., Dual effect of beta-adrenergic receptors on mitogen-activated protein kinase. Evidence for a beta gamma-dependent activation and a G alpha s-cAMP-mediated inhibition, J Biol Chem, 270 (1995), 25259-65.

115. Ciardiello, F., and Tortora, G., Interactions between the epidermal growth factor receptor and type I protein kinase A: biological significance and therapeutic implications, Clin Cancer Res, 4 (1998), 821-8.

116. Wheeler, AP., Hardie WD, Bernard GR., The role of cyclooxygenase products in lung injury induced by tumor necrosis factor in sheep, Am Rev Respir Dis, 145 (1992), 632-9.

117. Dascal N, Schreibmayer W, Lim NF, Wang W, Chavkin C, DiMagno L, Labarca C, Kieffer BL, Gaveriaux-Ruff C, Trollinger D, et al., Atrial G protein-activated K+ channel: expression cloning and molecular properties, Proc Natl Acad Sci U S A, 90 (1993), 10235-9.

118. Lim, NF., Dascal, N., Labarca, C., Davidson, N., Lester, HA., A G protein –gated K channel is activated via β2-adrenergic receptors and $G_{\beta\gamma}$ subunits in Xenopus oocytes, J Gen. Physiol, 105 (1995), 421-439.

119. Dascal, N., Signaling via the G-protein activated K^{+1} channels, Cell. Signal., 9 (1997), 551-573.

120. Isomoto, S., Kondo C., Kurachi Y., Inwardly rectifying potassium channels: their molecular heterogeneity and function, Jpn J Physiol, 47 (1997),11-39.

121. Mullner, C., Vorobiov, D., Bera, A.K., Dascal, N, Schreibmayer, W. Heterologous facilitation of G protein-activated K^{+1} channels by β-adrenergic stimulation via c-AMP-dependent protein kinase, J. Gen. Physiol., 115 (2000), 547-557.

122. Rogalski, SL., Cyr, C., Chavkin, C., Activation of the endothelin receptor inhibits the G protein-coupled inwardly rectifying potassium channel by a phospholipase A2-mediated mechanism, J Neurochem, 72 (1999) 1409-16.

123. Kim, D., and Pleumsamran, A., Cytoplasmic Unsaturated Free Fatty Acids Inhibit ATP-dependent Gating of the G Protein–gated K+ Channel, J Gen. Physiol, 115 (2000), 287-304.

124. Lohberger, B., Groschner, K., Tritthart, H., Schreibmayer, W., IK.ACh activation by arachidonic acid occurs via a G-protein-independent pathway mediated by the GIRK1 subunit, Pflugers Arch,441 (2000):251-6.

125. Rogalski, SL., Chavkin, C., Eicosanoids inhibit the G-protein-gated inwardly rectifying potassium channel (Kir3) at the Na+/PIP2 gating site, J Biol Chem, 276 (2001) 14855-60.

126. Kim, D., and Bang, H., Modulation of rat atrial G protein-coupled K+ channel function by phospholipids, J Physiol, 517 (1999), 59-74.

127. Kim, D, Sladek CD, Aguado-Velasco, C, Mathiasen JR., Arachidonic acid activation of a new family of K+ channels in cultured rat neuronal cells, J Physiol,484 (1995):643-60.

128. Terzic A, Tung RT, Inanobe A, Katada T, Kurachi Y., G proteins activate ATP-sensitive K+ channels by antagonizing ATP- gating, Neuron, 12(1994), 885-93.

129. Yamada, M, Terzic A, Kurachi Y.,Regulation of potassium channels by G-protein subunits and arachidonic acid metabolites, Methods Enzymol,238 (1994),394-422.

130. Stringer, BK., Cooper AG, Shepard, SB., Overexpression of the G-protein inwardly rectifying potassium channel 1 (GIRK1) in primary breast carcinomas correlates with axillary lymph node metastasis, Cancer Res, 61(2001), 582-8.

131. Schoots, O., Voskoglou, T., Van Tol, H.M., Genomic organization and promoter analysis of the human G-protein-coupled K+ channel Kir 3.1 (KCNJ3/HGIRK1). Genomics 39, (1997), 279-288.

132. McPherson, K, Steel, CM., Dixon JM.,ABC of breast diseases. Breast cancer-epidemiology, risk factors, and genetics, BMJ, 321 (2000), 624-8.

133. Bange, J., Zwick, E., Ullrich, A., Molecular targets for breast cancer therapy and prevention. Nat. Med, 7 (2001), 548-552.

134. Favoni, RE., and de Cupis, A., Steroidal and nonsteroidal oestrogen antagonists in breast cancer: basic and clinical appraisal. TIPS, 19 (1998), 406-415.

135. Bunone, G., Briand, PA., Miksicek, RJ., Picard, D., Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation, EMBO J, 15 (1996), 2174-2183.

136. Pietras, RJ., Arboleda, J., Reese, DM., Wongvipat, N., Pegram, MD., Ramos, L., Gorman, CM., Parker, MG., Sliwkowski, MX., Slamon, DJ., HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells, Oncogene,10 (1995), 2435-46.

137. Kato, S., et al., Activation of the Estrogen Receptor Through Phosphorylation by Mitogen-Activated Protein Kinase, Science, 270 (1995), 1491-94.

138. Mueller, H., Loop, P., Liu, R., Wosikowski, K., Kueng W, Eppenberger U., Differential signal transduction of epidermal-growth-factor receptors in hormone-dependent and hormone-independent human breast cancer cells, Eur J Biochem, 221(1994),631-7.

139. Biscardi, JS., Belsches AP, Parsons, SJ., Characterization of human epidermal growth factor receptor and c-Src interactions in human breast tumor cells, Mol Carcinog, 21 (1998), 261-72.

140. Clocca, DR., and Vargas Rolg LM., Estrogen receptors in human target tissues: biological and chemical implications. Endoc Rev, 16 (1995), 35-62.

141. Vladusic, EA., Hornby, AE., Guerra-Vladusic, FK, Lakins J, Lupu R., Expression and regulation of estrogen receptor beta in human breast tumors and cell lines, Oncol Rep,7(2000),157-67.

142. El-Tanani, M., and Green, C., Two separate mechanisms for ligand independent activation of the estrogen receptor, Mol Endocrinol, 11 (1997), 928.

143. Iwao, K., Miyoshi, Y., Egawa, C., Ikeda, N., Tsukamoto, F., Noguchi S., Quantitative analysis of estrogen receptor-alpha and -beta messenger RNA expression in breast carcinoma by real-time polymerase chain reaction, Cancer, 89 (2000), 1732-8.

144. Reddy, N., Everhart, A., Eling T, Glasgow W., Characterization of a 15-lipoxygenase in human breast carcinoma BT-20 cells: stimulation of 13-HODE formation by TGF alpha/EGF, Biochem Biophys Res Commun, 231 (1997), 111-6.

145. Scherer, RW., and Breitwieser GE., Arachidonic acid metabolites alter G protein-mediated signal transduction in heart. Effects on muscarinic K+ channels, J Gen Physiol, 96 (1990), 735-55.

146. Hallak, H., Muszbek, L., Laposata, M., Belmonte., E., Brass, LF., Manning, DR., Covalent binding of arachidonate to G protein alpha subunits of human platelets, J Biol Chem, 269 (1994), 4713-6.

147. Lo, C., Fu, M., Lo, R., Cryer, G., Cyclooxygenase gene activation is regulated by c-AMP, Shock, 13 (2000), 41-51.

148. Dennis, EA., Rhee, SG., Billah, MM., Hannun, YA., Role of phospholipase in generating lipid second messengers in signal transduction, FASEB J, 5(1991):2068-77.

149. Bandyopadhyay, GK., Hwang, S., Imagawa, W., Nandi, S., Role of polyunsaturated fatty acids as signal transducers: amplification of signals from growth factor receptors by fatty acids in mammary epithelial cells, Prostaglandins Leukot Essent Fatty Acids, 48(1993):71-8.

150. Naor, Z., Is arachidonic acid a second messenger in signal transduction, Mol. Cell. Endocr. 80 (1991), C181.

151. Elder, DJ., Halton, DE., Hague A, Paraskeva, C., Induction of apoptotic cell death in human colorectal carcinoma cell lines by a cyclooxygenase-2 (COX-2)-selective nonsteroidal anti-inflammatory drug: independence from COX-2 protein expression. Clin Cancer Res, 3 (1997), 1679-83.

152. DuBois, RN., Gupta, R., Brockman, J., Reddy, BS., Krakow SL, Lazar MA., The nuclear eicosanoid receptor, PPAR gamma, is aberrantly expressed in colonic cancers, Carcinogenesis, 19(1998), 49-53.

153. Hatala, MA., Rayburn J, Rose DP., Characterization of phospholipase A2 activity in MDA-MB-435 human breast cancer cells, Cancer Lett. 72 (1993), 31-7.

154. Williams, CS., Mann, M., DuBois, RN. The role of cyclooxygenases in inflammation, cancer and development, Oncogene, 18 (1999), 7908-7916.

155. DuBois, RN., Radhika A, Reddy BS, Entingh AJ., Increased cyclooxygenase-2 levels in carcinogen-induced rat colonic tumors. Gastroenterology, 110 (1996), 1259-62.

156. Tsujii, M, Kawano S, DuBois, RN., Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential, Proc Natl Acad Sci U S A, 94 (1997), 3336-40.

157. Rikitake, Y., Hirata, K., Kawashima, S., Takeuchi S., Shimokawa Y, Kojima Y, Inoue, N., Yokoyama, M., Signaling mechanism underlying COX-2 induction by lysophosphatidylcholine. Biochem Biophys Res Commun, 281 (2001), 1291-7.

158. Chang, L., Karin, M., Mammalian MAP kinase signaling cascades. Nature, 410 (2001), 37-42.

159. English, J., et al., New insights into the control of MAP kinase pathway, Exp. Cell Res, 253 (1999), 255-270.

160. Treisman, R., Regulation of transcription by MAP kinase cascades. Curr. Opin. Cell Biol, 8 (1996): 205.

161. Cobb, MH., MAP kinase pathways. Prog. Biophys. Mol. Biol, 71 (1999), 479-500.

162. Karin, M., Signal transduction from the cell surface to the nucleus through the phosphorylation of transcription factors, Curr Opin Cell Biol, 6 (1994), 415-24.

163. Treisman, R., Regulation of transcription by MAP kinase cascades, Curr Opin Cell Biol, 8 (1996), 205-15.

164. Guan, Z., ShaAvhree Y. Buckman, Alice P. Pentland, Dennis J. Templeton, and Aubrey R. Morrison., Induction of Cyclooxygenase-2 by the Activated MEKK1 →SEK1/MKK4→ p38 Mitogen-activated Protein Kinase Pathway, J. Biol. Chem. 273 (1998), 12901-12908.

165. Blank, JL., Pär Gerwins, Elicia M. Elliott, Susan Sather, and Gary L. Johnson., Molecular Cloning of Mitogen-activated Protein/ERK Kinase Kinases (MEKK) 2 and 3, J. Biol. Chem. 271 (1996), 5361-5368.

166. Masferrer, JL., Leahy, KM., Koki, AT., Zweifel, BS., Settle, SL., Woerner, BM., Edwards, DA., Flickinger, AG., Moore, R. J., Seibert, K., Antiangiogenic and Antitumor Activities of Cyclooxygenase-2 Inhibitors. Cancer Res, 60 (2000), 1306-1311.

167. Fulton, AM., Zhang, SZ., Chong. YC., Role of the prostaglandin E2 receptor in mammary tumor metastasis. Cancer Res, 51 (1991), 2047-2050.

168. Nikolic, D., and van Breemen, RB., 2001. DNA oxidation induced by COX-2, Chem Res Toxicol, 14 (2001), 351-58.

169. Tardieu, D., Jaeg, JP., Deloly, A., Corpet, DE., Cadet J, Petit, CR., The COX-2 inhibitor nimesulide suppresses superoxide and 8-hydroxy-deoxyguanosine formation, and stimulates apoptosis in mucosa during early colonic inflammation in rats, Carcinogenesis, 21(2000): 973-6.

170. Nantel, F., Marullo, S., Krief, S., AD Strosberg, and M Bouvier, Cell-specific down-regulation of the beta 3-adrenergic receptor, J. Biol. Chem. 269 (1994), 13148-13155.

171. Daaka, Y., Luttrell, LM., Ahn, S., Della Rocca, GJ., Ferguson, SG., Caron, MG., Lefkowitz, RJ., Essential Role for G Protein-coupled Receptor Endocytosis in the Activation of Mitogen-activated Protein Kinase, J. Biol. Chem. 273 (1998), 685-688.

172. Ferguson, SS., Downey, WE 3rd., Colapietro, AM., Barak, LS., Menard, L., Caron, MG., Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization, Science, 271 (1996), 363-6.

173. Benovic, JL., Bouvier, M., Caron, MG., Lefkowitz, RJ.,Regulation of adenylyl cyclase-coupled beta-adrenergic receptors, Annu Rev Cell Biol, 4 (1988), 405-28.

174. Benovic, JL., DeBlasi, A., Stone, WC., Caron, MG., Lefkowitz, RJ., Beta-adrenergic receptor kinase: primary structure delineates a multigene family, Science. 246 (1989), 235-40.

175. Lefkowitz, RJ, Hausdorff, WP., Caron, MG., Role of phosphorylation in desensitization of the beta-adrenoceptor, Trends Pharmacol Sci, 11 (1990):190-4.

176. Boulet, AP., Fortier, MA., Lambert, RD., The embryo influences adenylate cyclase activity and hormonal response in rabbit myometrium during early pregnancy, Life Sci, 43 (1988),1653-62.

177. Marchetti, B., Fortier, MA., Poyet P, Follea N, Pelletier G, Labrie, F., Beta-adrenergic receptors in the rat mammary gland during pregnancy and lactation: characterization, distribution, and coupling to adenylate cyclase, Endocrinology, 126 (1990), 565-74.

178. Loizzi, RF., Cyclic AMP changes in guinea pig mammary gland and milk, Am J Physiol, 245 (1983), E549-54

179. Draoui, A., Vandewalle, B., Hornez, L., Revillion, F., Lefebvre, J., Beta-adrenergic receptors in human breast cancer: identification, characterization and correlation with progesterone and estradiol receptors, Anticancer Res, (1991), 677-80.

180. Ciardiello, F., Tortora, G., Kim, N., T Clair, S Ally, DS Salomon, and YS Cho-Chung, 8-Chloro-cAMP inhibits transforming growth factor alpha transformation of mammary epithelial cells by restoration of the normal mRNA patterns for cAMP-dependent protein kinase regulatory subunit isoforms which show disruption upon transformation, J. Biol. Chem, 265 (1990), 1016-1020.

181. Hagiwara, M., Brindle, P., Harootunian, A., Armstrong R, Rivier J, Vale W, Tsien R, Montminy, MR., Coupling of hormonal stimulation and transcription via the cyclic AMP-responsive factor CREB is rate limited by nuclear entry of protein kinase A, Mol Cell Biol, 13 (1993), 4852-9.

182. Bertin, B., Jockers, R., Strosberg, AD., Marullo, S., Activation of a beta 2-adrenergic receptor/Gs alpha fusion protein elicits a desensitization-resistant cAMP signal capable of inhibiting proliferation of two cancer cell lines, Receptors Channels, 5 (1997), 41-51.

183. Taylor, SS., Buechler, JA., Yonemoto, W., cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes, Annu Rev Biochem, 59 (1990), 971-1005.

184. Strobl, JS., Wonderlin, WF., Flynn, DC., Mitogenic signal transduction in human breast cancer cells, Gen. Pharmac, 26 (1995), 1643-54.

185. Houge, G., Cho-Chung, YS., Doskeland, SO., Differential expression of cAMP-kinase subunits is correlated with growth in rat mammary carcinomas and uterus, Br J Cancer, 66 (1992),1022-9.

186. Mitra, SP., and Carraway, RE., Synergistic effects of neurotensin and beta-adrenergic agonist on 3,5-cyclic adenosine monophosphate accumulation and DNA synthesis in prostate cancer PC3 cells, Biochem Pharmacol, 57 (1999), 1391-7.

187. Slotkin, TA., Zhang, J., Dancel, R., Garcia, SJ., Willis, C., Seidler, FJ., Beta-adrenoceptor signaling and its control of cell replication in MDA-MB-231 human breast cancer cells, Breast Cancer Res Treat, 60 (2000):153-66.

188. Daaka, Y., Luttrell, LM., Lefkowitz, RJ., Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A, Nature, 390 (1997), 88-91.

189. McPhee, JC., Dang, YL., Davidson, N., Lester, HA., Evidence for a Functional interaction between Integrins and G Protein-activated Inward Rectifier K+ Channels, J. Biol. Chem, 273 (1998), 34696-34702.

VITA

Yavuz Cakir was born in Kayseri, Turkey in 1969. He graduated from Faculty of Veterinary Medicine at the University of Ankara, Turkey with a Doctorate of Veterinary Medicine in 1993. Following graduation, he practiced in small animal clinic for a short time. He won the National Exam done by Turkish Government to pursue graduate education in the US in 1994. He came to US in 1995 and got his Master of Science degree from Department of Veterinary Physiology and Pharmacology at The Ohio State University, Columbus, OH in 1997. He went back to home to get married his wife Iklime. He came back to US to pursue his doctoral degree in 1998. He enrolled in the Comparative and Experimental Medicine Program at the University of Tennessee to study in the area of experimental oncology for his doctoral research. In 2000, he received Graduate Fellowship from Department of Comparative and Experimental Medicine during his doctoral research.

Dr. Cakir has two great little sons, Emre and Enes, who gave him the encouragement and support he needed during his studies.