MECHANISTIC INVESTIGATION OF TOLFENAMIC ACID, BETULINIC ACID, AND ASPIRIN IN ANTI-CANCER THERAPY

A Dissertation

by

XINYI LIU

Submitted to the Office of Graduate Studies of Texas A&M University in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

August 2011

Major Subject: Biochemistry
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Approved by:

Chair of Committee, Stephen H. Safe
Committee Members, Gary R. Kunkel
David O. Peterson
James C. Sacchettini
Head of Department, Gregory D. Reinhart

August 2011

Major Subject: Biochemistry
ABSTRACT

Mechanistic Investigation of Tolfenamic Acid, Betulinic Acid, and Aspirin in Anti-Cancer Therapy.

(August 2011)

Xinyi Liu, B.MS., Chongqing Medical University

Chair of Advisory Committee: Dr. Stephen H. Safe

Tolfenamic acid (TA), betulinic acid (BA) and acetylsalicylic acid (aspirin) are anticancer drugs, and Sp1, Sp3, Sp4 (Sps) transcription factors and growth factor 2 (EGFR2, HER2 / ErbB2) are important molecular markers in cancer cells. In this study, the molecular mechanisms by which these anticancer drugs target downregulation of Sps and ErbB2 were investigated in breast cancer cells.

TA inhibits growth of ErbB2-overexpressing BT474 and SKBR3 breast cancer cells by inhibiting ErbB2 expression. TA downregulates ErbB2 mRNA expression and promoter activity, and TA also decreased expression of the YY1 and AP-2 transcription factors that are required for basal ErbB2 expression. These effects were accompanied by decreased ErbB2-dependent kinase activities, induction of p27, and decreased expression of cyclin D1. In addition, TA also inhibited tumor growth in BT474 cells orthotopic mouse model.
However, Sp proteins were not the major target of TA in these breast cancer cells.

BA inhibits growth of ErbB2–overexpressing BT474 and MDA-MB-453 and induced apoptosis in these cells. BA induced proteasome-independent downregulation of specificity protein (Sp) transcription factors Sp1, Sp3, Sp4 and Sp-regulated genes, and BA also decreased expression of ErbB2, ErbB2-regulated kinases and YY1. Knockdown of Sp1, Sp3, Sp4 and their combination by RNA interference was accompanied by decreased expression of ErbB2, YY1. BA-dependent repression of Sp1, Sp3, Sp4 and Sp regulated genes was due in part to induction of the Sp repressor ZBTB10 and downregulation of microRNA-27a (miR-27a) which constitutively inhibits ZBTB10 expression, and these effects is dependent on activation of the cannabinoid 1 (CB1) and CB2 receptors.

Aspirin inhibits growth and induces apoptosis in BT474 and MDA-MB-453 breast cancer cells. Aspirin downregulated expression of ErbB2, Sp1, Sp3, Sp4 and Sp-regulated genes, and these effects were inhibited in part by proteasome inhibitor lactacystin and phosphatase inhibitors. Aspirin-induced downregulation of Sp proteins are reversed in cells transfected with an oligonucleotide (siMKP5) that knocks down MKP5 by RNA interference, demonstrating for the first time a linkage between a drug-induced phosphatase (MKP-5) and Sp downregulation.

These results suggest that TA, BA and aspirin represent novel and promising new anticancer drugs for cancer treatment by targeting Sp proteins and ErbB2 oncogene.
DEDICATION

To my parents, Changming Liu and Qingkang Zhao, for their always being there, and unconditional support and love

To my husband, Guang Chen, for his love, care and support

To my beloved son, Andy Chen, for the endless happiness he gives me
ACKNOWLEDGEMENTS

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1. INTRODUCTION

1.1 Cancer

1.1.1 Cancer statistics

Cancer is the second leading cause of death after heart disease in the United States and for individuals between 40 and 79 years of age, cancer is the leading cause of death. Approximately 1.53 million new cases of cancer will be diagnosed in 2010 and 0.57 million people will die from this disease in the United States. The three most common tumor types among men are cancers of the prostate, lung and bronchus, and colorectum, and the cancers of breast, lung and bronchus, and colorectum are the 3 most commonly diagnosed tumor types among women. Cancers of the lung and bronchus, prostate, breast, and colorectum account for about 50% of all cancer deaths among men and women [1].

1.1.2 Causal factors of cancer

Cancer is not associated with a single cause. Multiple factors including tobacco, infectious organisms, chemicals, radiation, inherited mutations, hormones, immune conditions, and metabolically-mediated mutations have been identified as risk factors for the development of cancer (Table 1) [2].

Cigarette smoking is one of the major risk factors for development of cancer. Almost one-third of all cancers are related to smoking even though tobacco consumption has decreased in recent years. The incidence of lung can-
cancer has increased rapidly in smoking populations particularly among individuals who started smoking at an early age and have continued smoking throughout their adult years [2]. Smoking is a risk factor for cancers of the lung, pancreas, bladder, kidney, larynx, mouth, pharynx and oesophagus, and there is also evidence indicating that smoking may contribute to development of stomach, liver and probably cervical cancers [3,4]. Different populations have different susceptibilities to various smoking-related diseases. For example, smoking causes more liver cancer and less heart disease in China than in the USA [5].

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<td>Occupational or environmental conditions</td>
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<tr>
<td>Radiation</td>
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<tr>
<td>Infectious organisms</td>
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Diet and overweight can also affect cancer development. There is evidence showing that folate and aspirin reduce colon cancer incidence [6], and foods containing carotenoids, vitamins C and E decrease the incidence of some cancers. Cruciferous vegetables containing anticancer agents such as indole-3-carbinol (I3C) reduce colon, breast and pancreatic cancer risk [7]. Obese men and women are at highest risk for post-menopausal breast cancer, cancers of the endometrium, and gall-bladder and kidney compared to individuals in the “normal” weight range [8,9]. It has been suggested that approximately one-third
all cancers are associated with diet and obesity and these can be prevented in part by dietary changes [10].

Reproductive and hormonal factors primarily affect the incidence of breast and ovarian cancers, and both endogenous and exogenous hormones account for these effects [11,12]. For example, hormone replacement therapy and oestrogens administered as oral contraceptives increase incidence of breast cancer; increased parity and oral contraceptives lower the incidence of endometrial and ovarian cancers. Later age at first childbirth, lower parity and shorter periods of breastfeeding are the major reasons that women in developed countries have a higher risk of breast cancer compared to women in developing countries [13,14].

Epidemiology studies in the past two decades have demonstrated that infectious pathogens including viruses, bacteria and parasites are important risk factors for cancer. For example, it has long been recognized that hepatitis-B virus contributes to liver cancer, and recently it was reported that smoking synergistically enhanced hepatitis-induced hepatocellular carcinomas [15]. A major factor for development of stomach cancer is Helicobacter pylori, a gastric bacteria that causes gastric ulcers [15]. Human papillomaviruses can also cause anogenital, skin, and neck cancers [16].

Inherited genetic alterations have also received attention as important factors that influence development of cancer. Many studies show gene polymorphisms that are involved in mutagen metabolism strongly influence
cancer incidence. For example, in workers exposed to high levels of certain aromatic amines, the N-acetyltransferase (NAT2) slow acetylator phenotype confers a higher risk of bladder cancer [17]. Mutations in glutathione S-transferase genes are associated with increased lung cancer risk [18], and polymorphisms in oncogenes and tumor-suppressor genes also increase cancer risk. For example, a single nucleotide polymorphism at I1307K in the APC gene doubles the risk of colon cancer [19].

1.1.3 Tumor progression, oncogenes and tumor-suppressor genes

Human carcinogenesis is a multi-stage process, which can be divided into three basic steps, namely, initiation, promotion, and progression [20] (Fig. 1). In the initiation stage, pre-malignant lesions such as dysplasia and hyperplasia can be found in various organs prior to formation of malignant tumors. Environmental factors such as viral infections can induce polyclonal expansion of hyperplastic cells, and genetic alterations induce monoclonal expansion of the cells to give pre-malignant lesions. Subsequently, pre-malignant cells are converted into malignant cells of clonal origin which can form a primary tumor after accumulation of genetic alterations. This is called the promotion stage. The converted cells are not responsive to growth inhibitory factors and they expand into surrounding tissues; however, these cells are not invasive or metastatic at early stages of primary tumor formation. However, in the progression stage, primary tumors accumulate genetic alterations that result in formation of invasive and metastatic cells leading to the appearance of new clones. These highly
metastatic cells exhibit more gene alterations than non-metastatic cells [21] (Fig. 1).

Genetic studies of human cancers suggest that multiple gene alterations occur during cancer progression and these include changes in expression of oncogenes and tumor-suppressor genes. If mutated or highly expressed, an oncogene can transform a normal cell into a tumor cell. A proto-oncogene is a normal gene that can be converted into an oncogene due to mutations or overexpression. Proto-oncogenes are involved in regulation of cell growth and differentiation, wound healing, regeneration of the liver, growth factor-induced stimulation of cells, and embryogenesis. Proto-oncogenes encode for various growth factors, growth factor receptors, tyrosine kinases, regulatory proteins in signal transduction, and nuclear regulatory proteins[22] (Table2).

The mechanisms that activate proto-oncogenes to cancer-causing oncog-
enes can be classified as quantitative and qualitative. The quantitative mechanism is associated with gene amplifications, which result in increased expression of a proto-oncogene and its corresponding protein product. For example, amplification of the N-myc proto-oncogene has been linked to a number of human neuroblastomas, retinoblastomas, small cell lung carcinomas, and astrocytomas [23]; ErbB2 amplifications are associated with mammary carcinomas, salivary adenocarcinomas, and gastric adenocarcinomas [24]. The qualitative mechanism is due to DNA rearrangements, which lead to changes in nucleotide sequences that are pivotal for the activities of a gene. This mechanism may involve a reduction or increase in chromosome numbers, point mutations, deletions, translocations, inversions, isochromosomes, and ring chromosomes [25]. For example, as a consequence of a chromosomal rearrangement, c-myc and c-abl are activated by structural alterations in Burkitt's lymphoma and chronic myelocytic leukemia, respectively [26,27]. Activation of the ras oncogene due to a structural mutation was the first example of an activated proto-oncogene in human tumors. The ras proto-oncogene family is

<table>
<thead>
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<tr>
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<td>Growth factor receptor with tyrosine kinase activity</td>
</tr>
<tr>
<td>src, abl, lck, yes</td>
<td>Tyrosine kinase</td>
</tr>
<tr>
<td>Ha-ras, K-ras, N-ras, gsp, gip</td>
<td>Regulatory protein in signal transduction</td>
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<tr>
<td>Mos, raf</td>
<td>Serine/threonine kinase</td>
</tr>
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<td>Myc, myb, fax, c-jun, rel</td>
<td>Nuclear regulatory protein</td>
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</table>

Table 2. Proto-oncogenes and their functions.
interesting due to their importance in the development of many types of tumors. The K-, H-, and N-ras oncogenes are overexpressed and have point-mutations in more human tumors than any other oncogene. For example, a G35A point mutation of the K-ras gene is found in lung cancers; an A182T point mutation of the H-ras gene is detected in liver cancer, and G37C and C181A point mutations of N-ras gene are observed in skin cancers [28-30]. All the point mutations activate the ras oncogenes. The Philadelphia chromosome is an example of proto-oncogene activated by a chromosomal translocation. In 90% of patients with chronic myeloid leukemia, a balanced reciprocal 9:22 translocation occurs. Chromosome 9 acquires a breakpoint within an intron of the ABL oncogene, the translocation joins most of the ABL sequence with BCR gene on chromosome 22 to create a fusion gene. This fusion gene produces an ABL gene protein product with high cell transforming activity [31].

Another important type of genetic alteration that leads to cancer is the inhibition of tumor-suppressor gene expression. The incidence of tumor-suppressor gene mutations is higher than that of oncogene mutations in tumors. A tumor-suppressor gene protects cells from transformation, but in contrast with oncogenes which require activation, genetic alterations that inactivate tumor-suppressor gene activities are important for tumor development. In addition, a recessive mutation in which both alleles of the gene are altered is required for genetic alterations of tumor-suppressor genes [32].
The Human retinoblastoma gene was one of the first examples showing the importance of tumor-suppressor genes in carcinogenesis. Molecular studies show that the Rb gene prevents cells from entering into G1/S phase, and therefore mutation of this gene promotes cell proliferation [33]. Retinoblastoma, an embryonic neural retinal tumor that occurs in young children has heritable and non-heritable forms. The heritable form is a result of a chromosome deletion in which a germ-line mutation occurs on chromosome 13 at the Rb-1 locus. However, the result of this mutation is recessive, and is not sufficient to generate a tumor. Therefore a second mutation is required to un-mask the effects of the original Rb-1 mutation. Two somatic mutations are required to occur at each Rb-1 locus in non-heritable cases [34].

The p53 gene is another example of tumor-suppressor gene that is frequently mutated in human tumors and is associated with loss of heterozygocity (LOH) in high percentage of colon (70%), breast (30%-50%), and lung cancers (50%). Loss of heterozygosity is observed when one of the alleles of a gene is already inactivated and loss of function of the other allele results from mutations. This term is usually associated with tumor-suppressor gene inactivation and LOH can lead to dysfunction of a normal tumor-suppressor gene and thereby enhance tumor formation. Mutations of the p53 gene are detected in leukemias, lymphomas, sarcomas, and neurogenic tumors. There are two major functions of the normal p53 gene and one of them is to kill cells which have undergone DNA damage. The other function of p53 is to decelerate cells from
passing through the G0/G1 to S phase and thereby decrease cell proliferation. A mutation in only one tumor suppressor gene is usually not sufficient for a cell to become malignant. For example, functional defects in both the p53 and RB genes are observed in a number of human cancers, including small-cell lung carcinoma and osteosarcoma [35]. Rb and p53 regulate different pathways that inhibit cell proliferation and mutations of both genes enhance the likelihood that cells will evade growth inhibition. In some cancers, more than 3 tumor-suppressor genes are mutated. For example, colorectal mutant cancer protein (MCC), adenomatous polyposis coli (APC), p53, and deleted in colorectal cancer (DCC) are tumor-suppressor genes mutated in some colorectal cancers [36] and several combinations of tumor suppressor gene mutations are detected in diverse human tumors.

1.2 Breast Cancer

1.2.1 Breast cancer statistics

Breast cancer occurs mainly in women, and is rarely observed in men. In the United States, breast cancer is the second most commonly diagnosed cancer in women and it is estimated that 1 in 8 women in the United States will develop this disease [37]. Breast cancer incidence increases with age and the lowest incidence rate is observed in women aged 20-24 (0.0014%). The highest incidence rate (0.44%) is in women aged 75-79 and the decrease in incidence in women over 80 has been attributed to decreased screening and incomplete detection [37]. Mortality from breast cancer for women in the United States is
higher than all other cancers except lung cancer. In 2010, approximately 200,000 and 1970 new cases of breast cancer will be diagnosed in women and men respectively, and about 39840 female and 390 males are expected to die from this disease in 2010.

Fortunately, the incidence rates of breast cancer decreased by 2% between 1999 and 2006. This decrease is due to the reduced use of hormone replacement therapy (HRT) by women, suggesting that HRT is a risk factor for breast cancer. In addition, mortality rates for breast cancer have been decreasing since 1990 and from 1990-1995, the rates decreased by 1.8% annually, and from 1995-1998 and 1998-2006, the annual incidence rates decreased by 3.3%, and 1.9% respectively. These decreases are due to increased awareness, improved therapies and earlier detection of tumors due to increased use of mammography [2].

Incidence and mortality rates of breast cancer differ by race and ethnicity. During 2002-2006, Caucasian women in the United States had a higher incidence (0.12%) of breast cancer than African American women (0.11%) whereas African American women have higher mortality rates (0.033%) than Caucasians (0.023%) during the same period. This suggests that compared to African- American women, Caucasians are more likely to develop breast cancer, but less likely to die from this disease. One possible explanation is that African American women may develop more aggressive breast tumors than Caucasian woman, but the reasons for these differences are unclear. Compared with
Caucasian and African-American women, women with Asian, Hispanic, and American Indian ethnicity have lower rates of breast cancer incidence and mortality and the lowest among these groups are Asian women (0.08% incidence and 0.01% mortality) [38].

1.2.2 Classification, grade and stage of breast cancer

1.2.2.1 Structure of mammary gland

A normal human mammary gland is composed of hollow cavities, namely alveoli, cuboidal cells which are responsible for milk secretion and are enclosed by myoepithelium. The alveoli assemble to form lobules which harbor a lactiferous duct that are connected to openings in the nipple. Oxytocin excretion in the brain induces the myoepithelial cells to contract and expel milk secreted from the alveoli to the lobule lumen and through the lactiferous duct to the nipple. Tissues linked to a single lactiferous duct constitute a “simple mammary gland”, and all mammary glands serving one nipple are called a “complex mammary gland”. Humans normally have two complex mammary glands in each breast, and 10-20 simple mammary glands in each complex mammary gland [39]. Other components of the breast include mammary epithelial cells extracellular matrix (ECM) containing myoepithelial basement membrane and connective tissue, adipocytes, and fibroblasts. These structures are essential for maintenance of the polarized morphology of the lactiferous ducts, to support mammary structure, and to maintain connections between the mammary epithelium and their global environment [40] (Fig. 2).
1.2.2.2 Histopathological classification of breast cancer

Breast cancer is primarily classified according to its histopathological appearance and can be divided into two major groups, noninvasive and invasive breast cancer. Noninvasive breast cancers include ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS). Invasive breast cancers include invasive ductal carcinoma (IDC), invasive lobular carcinoma (ILC), tubular carcinoma, and some types that are rarely seen in clinic such as medullary carcinoma, mucinous carcinoma, metaplastic carcinoma, invasive cribriform carcinoma, invasive papillary carcinoma, and invasive micropapillary carcinoma. There are also other types of breast cancer that do not fit into any of the two groups and these include inflammatory breast cancer, Paget's disease of the nipple, and phylloides tumors (Table 3).

Ductal carcinoma in situ (DCIS) accounts for about 15% of the new breast cancer cases in the United States and is the most common noninvasive breast cancer. DCIS is characterized as uncontrolled cell growth that is limited to the ductal structure of the breast [41] and DCIS is often classified as a precancerous change. However, others believe that any cell changes exceeding atypical hyperplasia should be considered as cancer [42]. DCIS is usually diagnosed by mammogram screening and treated with surgery or radiation. DCIS is not life-threatening and the prognosis is good: the five-year survival rate is almost 100% when detected and treated at an early stage [43]. However, the incidence of de-
Figure 2. Anatomy of breast (Adapted from [44]).
veloping invasive breast cancer in the same site of at the opposite breast is higher in women who have been previously diagnosed with DCIS compared to women without DCIS [45].

| Table 3. Histopathological classification of breast cancer |
|---------------------------------|-------------------|------------------|
| Noninvasive                     | Invasive          | Other types      |
| DCIS                            | IDC               | Inflammatory breast cancer |
|                                 | ILC               | Paget’s disease of the nipple |
|                                 | Tubular carcinoma |                               |
|                                 | Medullary carcinoma |                             |
|                                 | Mucinous carcinoma |                              |
|                                 | Metaplastic carcinoma |                           |
|                                 | Invasive cribriform carcinoma |                       |
|                                 | Invasive papillary carcinoma |                     |
|                                 | Invasive micropapillary carcinoma |                 |
| LCIS                            |                   | Phyllloides tumors |

Lobular carcinoma in situ (LCIS) is characterized by accumulation of abnormal cells in the milk-producing breast lobules. Under the microscope, the normal lobules are interrupted abruptly by abnormal lobule groups in which the cells are twice the size of normal lobules, and their nuclei are proportionally enlarged with an opaque cytoplasm [46]. Both the incidence and the risk of developing invasive cancer of LCIS are lower than those of DCIS. LCIS frequently harbors multiple lesions and is often detected in both breasts. Symptoms associated with LCIS cannot be detected by mammography and the diagnosis usually occurs after a breast biopsy for other reasons [47]. Typically,
LCIS is considered as a marker of increased risk for future development of invasive breast cancer. Thus, most women diagnosed with LCIS need to increase their surveillance have frequent physical examinations and a mammogram every 6 months. For LCIS patients with a family history of breast cancer, a mastectomy is recommended [48] whereas post-menopausal women with LCIS are treated with tamoxifen or a similar antiestrogenic drug such as raloxifene [49].

Invasive ductal carcinoma (IDC) accounts for about 80% of invasive breast cancer, and also is the most common (70%) type of breast cancer. Histopathological studies show that the tumor appears as a peri and intra canalicul fibroadenoma with a number of atypical epithelial changes varying from atypical ductal hyperplasia to DCIS [50]. In addition to the in situ changes, infiltrating groups of malignant ductal epithelial cells are found in the surrounding breast tissues. Inflammatory responses are apparent around the DCIS and invasive carcinoma [51] and since the cells are metastatic they can migrate to other tissues through the lymphatic system or bloodstream. IDC tumors can cause retraction of the nipple or skin, and can easily be felt in a physical exam as an irregular-shaped hard lump and these are readily detected by mammography [52]. Treatment of IDC includes surgery, chemotherapy, hormonal and radiation therapy. Seventy percent of women diagnosed with IDC choose breast-conserving surgical treatments such as lumpectomy for relatively small tumor; mastectomy is used for large tumors to remove the entire breast
and some lymph nodes and thereby decrease the chance for tumor recurrence [53]. Most women with IDC are treated with chemotherapy and/or hormonal therapy after the surgery. Chemotherapy removes tumor cells that are rapidly proliferating, and hormonal therapy kills cells that require hormones for growth and both treatments inhibit tumor metastasis. In some cases, radiation therapy is used to remove remnants of cancer cells in the surgical area. Twenty year-survival rates of IDC patients range from 50%-98% depending on the tumor size [54].

Invasive lobular carcinoma (ILC) accounts for 10% invasive breast cancer and is the second most common types of breast cancer. ILC begins in the milk-producing lobules and penetrates into the surrounding tissues and spreads to other parts of the body. About two thirds of women with invasive breast cancer are over 55 years of age, and ILC is often observed in older women (early 60s) compared to IDC [55]. Hormone replacement therapy (HRT) after menopause increases the risk of ILC. Microscopically, the tumor is formed by uniform rounded cells with light cytoplasm and large nuclei with single nucleoli. The tumor cells aggregate in the alveoli and are separated by collagen-rich fibrous tissues. IDC tumors pass though the lobules into the adipose tissues and can be observed in the skin [56]. Compared with IDC, ILC often attacks both breasts and is more difficult to identify by mammography. Treatment of ILC includes local and systemic therapy and local treatment includes surgery and radiation. Lumpectomy or mastectomy is based on tumor size, and radiation is
recommended after mastectomy if the tumor is larger than 5 cm and/or the lymph nodes are involved. Chemotherapy and hormonal therapy are used as a systemic treatment to destroy any cancer cells that remain or have metastasized [57].

Tubular carcinoma is a rare invasive breast cancer that accounts for about 1-2% of all breast cancer cases. The average age of diagnosis of tubular carcinoma ranges between mid-40s to late-60s [58]. Tubular carcinoma is considered as a subtype of IDC; however, the microscopic appearance of tumor cells is quite different. In this case, the cancer cells are regular and highly differentiated, arranged in well-defined tubules. Tubular carcinoma is usually confined in the milk ducts and invasion to surrounding tissues is less likely than observed for other types of breast cancers [59]. Surgery can be conducted to remove the cancer and affected lymph nodes and procedures include lumpectomy which removes small size tumors, and mastectomy to remove large tumors and affected lymph nodes. Adjuvant therapy includes chemotherapy, radiation and/or hormonal therapy. The main goal of chemotherapy and radiation is to kill any remaining cancer cells at the tumor site and cells that metastasize to other tissues. Hormonal therapy with tamoxifen blocks the growth promoting effects of estrogen and since almost all tubular carcinomas are estrogen receptor positive, tamoxifen is a highly effective treatment [60].
Other sub-types of invasive breast cancer are very rare. For example, medullary carcinoma accounts for less than 5-7% of all invasive breast cancers; mucinous carcinoma is less than 5% of all invasive breast cancers; metaplastic carcinoma represents less than 5% of all breast cancers; invasive cribriform carcinoma comprises 5-6% of invasive breast cancers; invasive papillary carcinoma accounts for less than 1-2% of invasive breast cancers; and the incidence of invasive micropapillary carcinoma is less than 3% of invasive breast cancers [61].

Other types of breast cancers such as inflammatory breast cancer, Paget’s disease of the nipple, and phylloides tumors cannot be classified into either noninvasive or invasive breast cancers, and inflammatory breast cancer accounts for only 2% of breast cancer. Unlike other breast cancers, inflammatory breast cancer usually appears as redness, swollen, tenderness, and blockage of the lymph vessels in the skin of breasts. This type of mammary cancer does not grow like a solid tumor and cannot be detected by mammography or ultrasound resulting in a relatively poor prognosis [62]. Paget’s disease of the nipple accounts for only 1% of all breast cancers and begins in the milk ducts and appears like mild scaling, pain, and itching of the nipple. Paget’s disease can be in situ or invasive, and the prognosis is very good when the foci are in situ. Phylloides tumors start from the periductal stromal cells and accounts for less than 1% of breast cancers [63]. Phylloides tumors can be benign, borderline, or malignant and are composed of benign epithelium and
connective tissue, which determine whether the tumor is benign, borderline, or malignant. Total mastectomy is often used for treatment of phylloides tumors [64].

1.2.2.3 Grade of breast cancer

Pathologists often use histological grades to identify the type of breast tumor and determine its prognosis. The most commonly used grading scheme is the Scarff-Bloom-Richardson system [65]. To determine the grade of a breast cancer, pathologists usually observe three features of the breast tumor under microscope. The first one is the frequency of cell mitosis, the second one is formation of cancers that consist of tubular structure, and the third one is nuclear pleomorphism, which accounts for changes of cell size and uniformity. A score ranging from 1 to 3 is assigned to each of the features; a lower score means slower cell growth, and a higher score indicates faster cell growth. Hence, the three scores are added to give a final score ranging between 3 and 9. A tumor with a final score between 3 and 5 is classified as a Grade 1 tumor, and is characterized by well differentiated tumor cells; a final score of 6 or 7 is considered to be a Grade 2 tumor, in which the cells are moderately differentiated; and a final score of 8 or 9 is a Grade 3 tumor with poorly differentiated cells. Necrosis is another factor that determines tumor grade [66]. When necrosis occurs in a high grade tumor close to the outside margin of breast, the tumor is more likely to recur after the treatment.
1.2.2.4 Classification according to receptor status

Biological messengers such as hormones bind to receptors on the surface, in the nucleus or in the cytoplasm of the cells. Breast cancers can also be classified to subtypes according to their expression of receptors. Three important receptors: namely, the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2 / neu / ErbB2), may or may not be expressed in breast cancer cells and tumors. Breast cancer cells are classified as ER positive (ER+), ER negative (ER-), PR positive (PR+), PR negative (PR-), HER2 positive (HER2+), and HER2 negative (HER2-). Based on receptor status, breast cancers can be classified into four molecular groups: (1) basal-like breast cancer, which is ER-, PR- and HER2- (triple negative, TN); (2) luminal A, in which tumors are ER+ and considered to be low grade; (3) luminal B, which are ER+ and high grade and (4) HER2+ tumors, in which HER2 is amplified.

1.2.2.4.1 Basal-like breast cancers

Basal-like breast tumors express genes that are typically found in the normal basal myoepithelial cells of the breast. These genes include vimentin, αB crystalline, fasin, p-cadherin and cavelolin1 and 2, and basal cytokeratins (CKs) such as CK5 / 6, CK14 and CK17 [67-69]. Since CK expression is one of the most important features of basal-like tumors, they have been used in almost all immunohistochemistry studies as markers to define basal-like tumors. Basal-like breast cancer is often observed in young individuals and accounts for 15% of all
breast cancers. Over 60% of basal-like breast carcinomas show expression of epidermal growth factor receptor (EGFR) and unusually high levels of genes associated with cell proliferation, such as Ki-67 and PCNA [70,71]. Mutations in p53 are observed in about 85% of basal-like breast cancers [72].

Basal-like breast carcinoma appears as high histological grade with a high percentage of cell mitosis and a central necrosis area which may reach the border of the tumor and infiltrate into lymphocytes [73]. Large portions of the tumors show typical ductal structures and occasional tubular foci. The tumor cells are usually pleomorphic with high nuclear-cytoplasmic ratio. Metaplastic features such as spindle cells and squamous cell metaplasia, and medullary characteristics are frequently observed in basal-like breast carcinomas, and almost all medullary breast carcinomas and over 90% of metaplastic breast carcinomas exhibit a basal-like phenotype [74,75].

Basal-like breast cancers exhibit an aggressive clinical behavior and poor outcomes, and may develop a locoregional and distant metastasis within the first five years after initial diagnosis. Theses tumors often metastasize to the brain and lung [76], and rarely to the bone and liver [77].

Frequently, basal-like breast cancer are considered to be triple-negative (TN) breast cancer because of the absence of ER, PR and HER2 expression in almost all basal-like breast cancers. Triple-negative breast cancers constitute 10-17% of all breast cancers, and the range depends on the standards used to assess receptor status [78,79]. Both basal-like breast cancer and triple negative
breast cancer preferentially affect young African-American women; both of the two tumor types usually exhibit a histological grade of 3, even though about 10% of triple-negative breast cancers are in grade 1 [80]; both of the two tumor types are aggressive and patients have a poor prognosis [81].

A major similarity between basal-like and triple-negative breast cancers is the occurrence of breast cancer 1(BRCA1) mutations. BRCA1 is a human tumor-suppressor gene and was identified in 1990 by King and coworkers [82] and cloned in 1994 [83]. The protein encoded by BRCA1 repairs damaged DNA and kills cells when the DNA damage cannot be repaired. Mutation of the BRCA1 gene leads to uncontrolled proliferation of cells with damaged DNA and this enhances tumor formation [84]. The protein product of BRCA1 is called breast cancer type 1 susceptibility protein. It binds to other proteins such as DNA damage sensors, signal transducers and tumor suppressors to form a multi-subunit protein complex, namely the BRCA1-associated genome surveillance complex (BASC) [85]. The BASC complex is involved in transcription, double-stranded break DNA repair, ubiquitination and transcriptional regulation. Many tumors with a BRCA1-mutation are triple-negative and also express basal CKs [86], and microarray profiling of RNAs from these tumors show that BRCA1 mutations exhibit basal-like tumor mRNA expression [87], suggesting similar causes and / or signaling pathways in the two subtypes.

Since basal-like and triple negative breast cancers exhibit many similarities, can they be combined as a single type of tumor? It has been
proposed by some investigators that the basal-like breast cancers are primarily triple-negative with respect to ER, PR and HER2 expression whereas this has been disputed by others [88]. Microarray-based expression profiling revealed that 15-54% of the basal-like subgroup of breast cancer expresses at least one of the ER, PR and HER2 receptors [89]. Furthermore, careful analyses of microarray-based expression profiles indicate that triple-negative breast cancer also includes other subtypes of breast tumors designated as normal-breast-like cancers. The so-called normal breast-like cancers are clustered with basal-like and are HER2+ / ER- [86]. Therefore, the basal-like and triple-negative breast tumor definitions cannot be simply combined to describe the same subtype of breast tumors due to their own distinctive characteristics.

Basal-like / triple-negative breast tumors are one of the most challenging subtypes of breast cancers, since the hormone receptors and HER2 are not expressed and these tumors can only be treated with cytotoxic anticancer drugs. [90]. Triple-negative breast tumors do not respond to existing targeted therapies such as endocrine therapy and monoclonal antibodies such as trastuzumab, or tyrosine kinase inhibitors. Persistent follow-up of triple-negative breast cancer cases indicates a poor prognosis of this subtype compared to patients with tumors expressing hormone receptors [91]. Chemotherapeutic agents used for treatment include doxorubicin, cyclophosphamide, methotrexate, paclitaxel, fluorouracil, epirubicin, docetaxel, vinorelbine, gemcitabine, capecitabine, and carboplatin. Ixabepilone, an epothilone analog which binds tubulin, stabilize
microtubules, arrest cell cycles, and cause subsequent apoptosis of cells, appears to be reasonably effective for treating triple-negative breast tumors and other metastatic breast cancers [88]. Some new targeted therapies are emerging for treating TN cancers. Histological studies of basal-like / triple-negative tumors indicate extensive glomeruloid microvascular proliferation. Therefore, angiogenesis inhibitors such as bevacizumab, a human monoclonal antibody for vascular endothelial growth factor (VEGF), show some success in treatment of the triple-negative subtype of breast cancer [92]. Another emerging targeted therapeutic agent is cetuximab, a human monoclonal antibody against EGFR, which is overexpressed in these tumors, and it has been used with limited success in treatment of the triple-negative breast cancer. [93]. However, single agents are minimally effective for treating triple-negative cancers and ongoing studies with drug combinations may be more successful.

1.2.2.4.2 Luminal breast cancers

Another subtype of breast cancer according to receptor status is hormone receptor positive breast cancers. Hormone receptors that are expressed in breast cancer cells include estrogen receptor (ER) and progesterone receptor (PR).

The ER responds to the hormone 17 β - estradiol (E2, estrogen), and ER is a member of the nuclear hormone receptor family, and functions as a ligand-activated transcription factor. ERα and ERβ are two forms of the receptor and are encoded by separate genes, ESR1 and ESR2, respectively [94]. In response
to estrogen, ER forms dimers which could include ER\(\alpha/\)ER\(\alpha\) and ER\(\beta/\)ER\(\beta\) homodimers, and ER\(\alpha/\)ER\(\beta\) heterodimers. ER\(\alpha\) and ER\(\beta\) have significant sequence homology, and both receptors contain five domains typical of nuclear receptors [95]. The N-terminal A/B domain exhibits ligand-independent transactivation activity. The DNA-binding domain (C) contain zinc finger motifs that bind to estrogen response element (ERE) in promoter regions of target genes and the D domain contains a flexible hinge region. The E domain, also known as ligand binding domain binds to estrogenic ligands and also interacts with coactivator and corepressor proteins. After ligand binding, the ER becomes transcriptionally active and this results in the induction of gene expression (Fig. 3). The function of the C-terminal F domain is not clear [96]. Although both ERs are extensively expressed in various tissue types, they have different expression patterns: ER\(\alpha\) is expressed in breast, ovarian stroma, endometrium and hypothalamus, while ER\(\beta\) is distributed in lungs, intestine, kidney, brain, bone, heart prostate, and endothelial cells [97].

Ligands activate ER-dependent genomic and non-genomic pathways. In the classical genomic pathway, estrogen receptors reside in the nucleus and after binding ligand the bound complex binds to EREs or other motifs in E2-responsive genes to activate transcription [94]. The ER also regulates transcription of genes that contain other motifs such as an AP-1 or GC-rich sites in which the ER binds fos/jun or Sp proteins respectively which are themselves
bound to DNA [98,99]. In non-genomic pathways, some estrogen receptors increased. This cross-talk between growth factors and estrogen receptors can r-

eside in the cell surface membrane and in lipid-rafts and caveolae where they may interact with growth factor receptors such as EGFR, HER2 and Insulin-like growth factor 1 (IGFR1). These cell surface-associated estrogen receptors can be rapidly activated when cells are exposed to estrogen [101]. The non-genomic ER pathway leads to rapid activation of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinases (PI3K) signaling [102]. Nuclear
estrogen receptors are phosphorylated by activated extracellular-signal-regulated kinase (ERK) and protein kinase B (AKT/PKB), and transcription activity and the stability of phosphorylated nuclear estrogen receptors are result in resistance to ER antagonists such as tamoxifen [103].

The progesterone receptor is a member of the steroid-receptor superfamily of nuclear receptors. It is also named nuclear receptor subfamily 3 (NR3C3). The PR is encoded by the PGR gene on chromosome 11 [104]. This single gene uses two different promoters and translational start sites to produce two isoforms, PR-A and PR-B. The PR protein also contains five domains, and the transcriptional activation function-3 (TAF-3) domain is only present in the amino acid terminal of the PR-B isoform [105]. The two isoforms of PR are functionally distinct. For example, PR-B induces proliferation of epithelial cells in the uterus in response to both progesterone and estrogen or estrogen alone, while the PR-A isoform inhibit estrogen-induced epithelium proliferation [106].

Hormone receptor positive breast cancers are also called luminal breast cancer. They have similar expression pattern as the luminal epithelium of the breast, in which luminal cyto-keratins 8/18, ER and ER activating genes such as LIV1 and cyclinD1 are expressed [89,107]. There are two subtypes within the luminal breast cancers, luminal A and luminal B. Luminal A subtype is characterized as expressing ER and PR, associated with low histological grade and cell proliferation rate, and good prognosis (>95% 5 year survival). Luminal B breast cancer is defined as expressing ER, PR and HER2 and often with high
histological grade; 50-70% of all breast cancers belong to the luminal subtype. Luminal A subtypes express more ER-associated genes such as GATA-3 and FOXA1, and less proliferative genes than luminal B subtype [108]. GATA-3 is a member of the GATA transcriptional factor family [109], and FOXA1 belongs to the “winged helix” transcriptional factor family [110]. FOXA1 binds to the condensed form of chromatin and increases chromatin accessibility to transcription factors such as ER to bind DNA and initiate transcription. GATA-3 cooperates with FOXA1 to open chromatin efficiently and increase accessibility of DNA regulatory elements. Cooperation among GATA-3, FOXA1 and ER is essential for development of mammary gland, and loss of control of this cooperation may be an indicator for ER positive breast cancers [111].

Although both luminal subtypes have good prognosis, luminal A has a better outcome than luminal B and this is related to responsiveness to therapy [86]. Luminal breast cancers usually have good responses to hormone therapy and poor response to chemotherapy. However, recent studies suggest that luminal A subtype may receive sufficient benefit from endocrine therapy alone, and luminal B subtype may be adequately treated with combination of endocrine therapy and chemotherapy, considering that luminal B cells express more proliferative genes and less ER-related genes than luminal A tumors [112]. Recent studies show that targeted therapy may also work for treating luminal breast cancers. Combination usage of bevacizumab, a VEGF antibody, with
paclitaxel, a mitotic inhibitor used in chemotherapy, may improve prognosis of luminal breast cancer patients [113].

1.2.2.4.3 HER2+ breast cancers

HER2 positive breast tumors account for 15%-20% of all breast cancers. Clinical detection of HER2 is conducted by immunohistochemistry (IHC) for HER2 overexpression or by fluorescence in situ hybridization (FISH) for HER2 gene amplification [114]. HER2 positive breast tumors often fall in high histological grades (grade2-3), and are associated with poor differentiation, high rate of cell proliferation, lymph-node involvement, and poor prognosis. The poor prognosis of HER2 positive breast cancer may due to its increased invasion, metastasis, and angiogenic activity [115]. HER2 overexpression is largely due to amplification at the DNA level, which is related to relapse and death even in cases of early-stage breast cancer [116]. Furthermore, HER2 is preferentially expressed in hormone receptor-negative breast cancer cells than hormone receptor-positive cases and this accounts for a decreased response of HER2 positive cells to hormonal regulation through cross-talk with the estrogen receptor complex [117,118] (Fig. 4). In fact, HER2 amplification has been shown to negatively correlate with estrogen receptor expression in a number of breast cancer cell lines, and estrogen decreases HER2 mRNA and protein levels in ER positive breast cells [119,120]. Since HER2 positive breast cancer exhibit minimal ER expression, they are not the good candidate endocrine therapy. The
Figure 4. The pathologic stage of a tumor (adapted from [121]).
most important and efficient treatment for this subtype to date is passive immunotherapy. Heceptin (trastuzumab), a humanized monoclonal antibody against the extracellular domain of HER2, is extensively used to inhibit growth of HER2-overexpressing cancer cells. It can be used before, after, or in combination with different chemotherapy agents [122]. (Details of ErbB2 targeted drugs will be elucidated at the end of chapter 1.2) When inflammation and invasion is observed in the tumor, overexpression of HER2 indicates a better prognosis, even though HER2 is usually a marker for poor outcome. In addition, HER2-responsive T-cells were identified in patients with HER2 positive tumors. This suggests a role for HER2 in immune-surveillance and this spurred an investigation of active immunotherapy for HER2 positive breast tumors [123]. HER2-directed vaccines have been generated and active immunotherapy strategies have been tested in animal studies and clinical trials [124]. Since proliferation of HER2 positive tumors depends on HER1 and HER2, tyrosine kinase inhibitors (TKIs) that specifically inhibit HER1 phosphorylation and function have also been developed. For example, ZD1839, one of the HER1 TKIs, can effectively inhibit growth of HER2-overexpressing cells with normal or low HER1 levels [125]. There are many other HER2-derived antibodies and neoadjuvant therapies for treating HER2 positive breast tumors and these will be discussed in more detail in a separate subsection of this Introduction.

Relationships between classification of receptor status and histological grade is shown in Figure 4.
1.2.2.5 Stages of breast cancers

Clinicians have developed a staging system to determine the extent of tumor development and metastasis. The clinical stage of a breast tumor is based on the physical exam, biopsy, and imaging test results. The pathologic stage of a tumor is based on the above tests and results of surgery which evaluates both the tumor and its spread to lymph nodes as indicated in Figure 4. Since it provides doctors with firsthand information on the extent of a cancer, the pathologist staging is more accurate than the clinical staging system. According to the American Joint Committee on Cancer (AJCC), the TNM system is the most common pathological staging system for assessing breast cancer. In the TNM system, T stands for tumor, which depicts the tumor size, and the extent that the tumor has spread within the breast and to surrounding organs; N stands for lymph nodes, which describes migration to lymph nodes; and M is for metastasis, which designates metastasis of the tumor to distant organs.

Additional numbers or letters are used after T, N, M; a number from 0 to 4 after the letter T defines the tumor size and extent of spreading to the skin or chest wall, and higher numbers correspond to an enhanced effect.

A number from 0 to 3 following the letter N describes the extent of cancer metastasis to lymph nodes. A number of 0 or 1 after the letter M indicates the extent of metastasis to distant organs.

In the T category, TX indicates that the primary tumor cannot be assessed, T0 suggests no evidence of a primary tumor, Tis is assigned to carci-
noma \textit{in situ} (DCIS, LCIS, or Paget disease), T1 designates a tumor smaller than 2 cm, T2 indicates a tumor between 2 and 5 cm, T3 indicates a tumor bigger than 5 cm, and T4 means a tumor of any size growing into the chest wall or skin. In the N category, NX means nearby lymph nodes cannot be assessed, N0 indicates that cancer has not been detected in nearby lymph nodes, N1 defines tumors spread to 1 to 3 axillary lymph nodes, N2 is defined as cancer that has spread to 4 to 9 axillary lymph nodes, and N3 is used to describe a cancer detected in more than 10 axillary lymph nodes with at least one area larger than 2 mm. In the M category, M0 means no distant spread of the tumor is observed in x-ray or physical examination, and M1 indicates tumor metastasis.

Once the TNM categories have been determined, stage grouping with information from each category is combined, and an overall stage number is assigned for each breast cancer patient (Table 4). According to the National Cancer Institute’s Surveillance, Epidemiology, and End Results (SEER) database, the 5-year survival rate of breast cancer patient classified in various stage is summarized in Table 4.

1.2.3 Breast cancer therapies

Significant improvements have been made in breast cancer therapies, and patients have many treatment options, including surgery, radiation therapy, chemotherapy, endocrine therapy, and targeted therapy and these are discussed in the following subsections.
1.2.3.1 Surgery and radiation therapy

Surgery is used for most patients to remove mammary tumors. In the early days of breast cancer treatment, radical mastectomy was used to remove the entire breast, pectoral muscles, all of the axillary lymph nodes, and the overlaying skin. More moderate surgical procedures termed modified radical mastectomy (MRM) are now used to remove the mammary tumors and most of the axillary lymph nodes; 5 year survival rates for patients who have undergone MRM is similar to that for women with radical mastectomy [126].

Breast-conserving surgery is a procedure that removes the cancer but leaves the breast intact and is widely prescribed for patients with early stage breast cancer, such as DCIS, or invasive breast cancer in stages I and II [127]. Breast-conserving surgery (lumpectomy / partial mastectomy) is normally followed by radiotherapy and this procedure preserves the breast structure and appearance compared to mastectomy. Radiation therapy following surgery eliminates microscopic tumors that remain after radiation [128].

Radiotherapy can also be used with mastectomy [129] and the National Cancer Institute recommends that patients with metastasis in more than four axillary lymph nodes undergo postmastectomy radiotherapy. Breast cancer is often metastasized to lung, liver, bone and brain, and radiation therapy is also applied to relieve the pain from the metastasis [130].

1.2.3.2 Chemotherapy

Antineoplastic chemicals are used in chemotherapies to treat malignant
tumors and these drugs are invariably cytotoxic compounds that impair mitosis and cause extensive cell damage and death [131]. An important principle of ch-

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emotherapy is that the cytotoxic chemicals effectively target rapidly-dividing cells and thus malignancies with high growth rates such as aggressive lymphomas and acute myelogenous leukemia are more sensitive to chemotherapy compared to tumors with lower growth rates such as indolent lymphomas and also normal tissues with even lower rates of cell proliferation [132]. Some solid tumors are also less responsive to chemotherapy, since cell division is relatively low and these are treated by surgery or radiation therapy.

Most cancers are currently treated with surgery or radiation therapy along with various combinations of anticancer drugs. Neoadjuvant chemotherapy in which chemotherapeutic drugs are used to decrease the size of the primary tumor prior to surgery is highly effective for treating some tumors and postoperative adjuvant chemotherapy is useful for preventing tumor recurrence and killing cancer cells that have metastasized [133].

Cytotoxic chemotherapeutic drugs include alkylating agents, anti-metabolites, microtubule disrupters derived from natural products, and topoisomerase inhibitors, all of which impair cell division or DNA synthesis. Alkylating agents include cisplatin, carboplatin, and oxaliplatin; these compounds alkylate nucleophilic groups and form covalent bonds with the amino, carboxyl, sulfhydryl, and phosphate moieties in macromolecules and this results in impaired cell function. Other alkylating agents such as mechlorethamine, cyclophosphamide, chlorambucil, and ifosfamide chemically modify DNA to disrupt cell function [134]. Anti-metabolites include purine
analogues (azathioprine, mercaptopurine, thioguanine), pyrimidine analogues (5-fluorouracil (5FU), floxuridine (FUDR), cytosine arabinoside), and antifolates such as methotrexate. These agents are structural mimics of purines, pyrimidines and folates which are required for DNA synthesis and the antimetabolites inhibit DNA synthesis during the S phase of the cell cycle and this blocks cell division [135].

Several naturally occurring plant alkaloids, terpenoids and their synthetic analogs disrupt microtubule function to block cell division. These include vinca alkaloids, Podophyllotoxin and taxanes [136]. Vinca alkaloids are derived from Madagascar periwinkle, a herbaceous perennial plant, and they bind to specific sites on tubulin to inhibit its assembly into microtubules. Podophyllotoxin can be isolated from American Mayapple, and this compound prevents cells from entering the G1 phase and DNA replication. Paclitaxel, a prototype of the taxane drugs, is derived from the bark of Pacific Tew tree, and taxanes can increase the stability of microtubules and this prevent chromosomes from separating during anaphase [137].

Topoisomerases maintain the normal topological structure of DNA, and inhibition of topoisomerases impairs proper DNA supercoiling and interferes with DNA replication and transcription [138]. Type I topoisomerase cuts one of the DNA strands causing relaxation, and the cut strand is then reannealed; Type II topoisomerase cuts both strand of the DNA, delivers another intact DNA helix through the broken end, and then reanneals the cut strands. Examples of type I
topoisomerase inhibitors include camptothecins such as irinotecan and topotecan; amsacrin, etoposide and etoposide are examples of type II topoisomerase inhibitors. These cytotoxic chemotherapeutic drugs function by killing rapidly dividing tumors cells and also normal cells that divide rapidly, such as cells in the bone marrow, digestive tract and hair follicles. This causes the common side effects observed in patients treated with these agents, namely, decreased blood cell production, inflammation of the digestive tract and hair loss [139].

1.2.3.3 Endocrine therapy

There are two major types of endocrine therapy used for treating hormone receptor positive breast cancers, namely, drugs inhibit estrogen-ER binding, and surgery or drugs that block ovarian production of hormones and these therapies are discussed in the following subsections.

1.2.3.3.1 SERMs and antiestrogens

Estrogen is a multi-functional hormone that regulates physiological functions in the breast, but also in other organs including ovary, bone, cardiovascular system and nervous system. Thus medications that interfere with ER function to inhibit breast cancer may also disrupt the normal action of estrogen in other organs and cause undesirable side effects. Therefore, selective estrogen receptor modulators (SERMs) have been developed for breast cancer therapy and these compounds selectively modulate ER action in specific organs [140]. A SERM can be an ER agonist in some tissues and
antagonist in others, and SERMs such as tamoxifen are also called mixed ER agonists / antagonists [141]. Fulvestrant (ICI-182780) is a SERM that is an ER antagonist in most if not all tissues and only endogenous estrogen is an ER agonist in all tissues.

Tamoxifen (Nolvadex) is a SERM that is an ER antagonist in mammary tumors and an agonist in most other tissues including the endometrium. Tamoxifen has been used as endocrine therapy for treatment of early and advanced ER+ breast cancer in both pre- and post-menopausal women [142]. Tamoxifen has also been approved by the Food and Drug Administration (FDA) for prevention of breast cancer in women at high risk of this disease, and to reduce the risk of breast cancer in the opposite site [143].

Tamoxifen is a substituted triphenylethylene derivative (Fig. 5) which has low affinity for the ER. Tamoxifen is metabolized in the liver by P450 isoforms CYP2D6 and CYP3A4 and its major metabolites, 4-hydroxytamoxifen and N-desmethyl-4-hydroxytamoxifen (endoxifen) have 30-200 times higher affinity for the ER compared to tamoxifen and 4-hydroxytamoxifen is considered to be the “active” metabolite [144]. The activity of SERMs as ER agonists or antagonists is dependent on several factors including the SERM-induced conformational changes in the ER and subsequent interactions with coactivators, corepressors and other nuclear cofactors and this will also depend on tissue-specific expression of these proteins [145]. In mammary tumors, tamoxifen metabolites interact with ER, bind DNA and recruit co-repressors such as nuclear receptor
co-repressor (NcoR) and silencing mediator of retinoid and thyroid receptors (SMRT) to inhibit transcription of estrogen-responsive genes [146]. In bone and uterus, where tamoxifen acts as an ER agonist, the DNA-bound ER complex recruits co-activators such as steroid receptor co-activators (SRCs), CREB-
binding protein (p300/CBP), p68 RNA helicase, and ribosomal protein L7/switch protein for antagonists (L7/SPA) [147-149], which are more highly expressed in bone and uterus compared to breast cancers. Protease digestion followed by analysis of ligand-bound ER showed that in breast tumors, tamoxifen induces a conformational change of ERα that is more favorable for recruiting co-repressors [150].

During the course of tamoxifen treatment, ER-positive breast tumors often develop resistance, which is frequently accompanied by loss of ER [151]. In a number of breast cancer cases, however, tamoxifen-resistant tumor cells remain ER+ and this may be due to increased metabolic deactivation of tamoxifen [152], increased cellular kinase activities [153], and activation of the P-glycoprotein multidrug-resistance efflux pump [154].

Given its activity as an ER agonist in the bone and uterus, tamoxifen is also used to maintain bone density [155], and to treat infertility in women with anovulatory disorders [156]. With exception of these beneficial estrogenic effects, tamoxifen treatment is associated with adverse side-effects, including minor symptoms of mood disturbance, weight gain, hot flashes and atrophic vaginitis, increased incidence of pulmonary embolism, stroke, and deep-vein thrombosis. Two other tamoxifen side-effects are an increased incidence of endometrial carcinoma and thromboembolic phenomena in postmenopausal but not in premenopausal women [157].
Raloxifene (LY 156758) is a newly developed SERM that has anti-estrogenic actions on the breast and uterus and estrogenic actions on bone. Unlike tamoxifen, primary applications of raloxifene are not for treating breast cancer, but for prevention of breast cancer, cardiovascular diseases and osteoporosis in postmenopausal women. Raloxifene binds ER with high affinity but has a shorter biological half-life and is less potent than tamoxifen [158]. Raloxifene has some cross-resistance with tamoxifen and is barely effective for treating tamoxifen-resistant breast tumors [159].

Since raloxifene acts as an antagonist in both breast and uterus, it reduces the risk of breast cancer without increasing incidence of endometrial cancer [160]. The results from Multiple Outcomes of Raloxifene Evaluation clinical trials show that raloxifene increases bone mineral density in the spine, hip and femoral neck, and decreases vertebral fracture incidence in postmenopausal osteoporotic women [161]. Raloxifene decreases levels of serum low-density lipoprotein (LDL) and decreases the incidence of coronary heart disease by 40% [162].

Arzoxifene (LY 353, 381) is a raloxifene analog that acts as an ER antagonist in mammary and uterine tissue but an ER agonist in bone. Arzoxifene has a longer biological half life and is more potent than raloxifene, and is more effective than raloxifene as an inhibitor of nitrosomethylurea induced rat mamm-
ary tumor formation [163]. Arzoxifene maintains bone density and lowers serum cholesterol more efficiently than raloxifene without causing uterotrophic effects [164].

ICI 182,780 (Fulvestrant) is a pure antiestrogen with no ER agonist activity. ICI 182,780 induces ER downregulation and is a second-line agent after the failure of tamoxifen for treating ER+ metastatic breast cancer in postmenopausal women. Fulvestrant is administered monthly as an injection and this drug exhibits less cross-resistance with tamoxifen compared to other SERMs. For example, tamoxifen-resistant MCF-7 breast cancers respond to ICI 182,780 [165].

1.2.3.3.2 Ovarian ablation and aromatase inhibitors

Inhibition of estrogen biosynthesis is also an important strategy for breast cancer therapy. The ovaries are the major site of estrogen synthesis in premenopausal women, and ovarian ablation was extensively used for treating breast cancer patients. Surgical ovariecotmy results in a permanent and immediate decrease in ovarian hormone production [166]. Due to the relatively high rate of surgical complications from ovariecotmy, current applications of laparoscopic surgery have significantly reduced the mortality and morbidity from the operation.

Radiation is another form of ovarian ablation, in which the treatment algorithms range from 450 cGy in one fraction to 2000 cGy in five or six fractions [166]. Radiation is a simple and safe approach for ovarian ablation with a
disadvantage of possible incomplete ablation and reversibility in some individuals.

During the past twenty years, medical ovarian ablation has also been extensively used and include chronic administration of analogs of luteinizing hormone-releasing hormone (LHRH, also known as gonadotropin-releasing hormone (GnRH)) and these include leuprolide and goserelin which cause temporary chemical castration [166]. Ovarian estrogen synthesis is controlled by the release of LHRH from the hypothalamus, which leads to production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in vertebrates in the pituitary gland and these gonadotropins stimulate the ovarian steroidogenesis (Fig. 6). Binding of LHRH agonists to pituitary gonadotropin receptors prevents binding of endogenous LHRH resulting in decreased secretion of gonadotropin from the pituitary gland and diminished steroid production in the ovaries. The major advantage of medical ovarian ablation is its noninvasiveness and reversibility. Comparative trials of goserelin with surgery and radiation have shown that the three strategies have similar effects in treating ER+ breast cancer patients [167], and the three approaches are often applied interchangeably.

In contrast to premenopausal women, the major areas for producing estrogen in postmenopausal women are peripheral adipose tissue and the adrenal gland where androgens are converted into estrogens. An important strategy for treating ER+ breast cancer in postmenopausal women involves the
Figure 6. Synthesis of estrogen and progesterone in premenopausal and postmenopausal women (Adapted from [168]).
use of aromatase inhibitors, which inhibits the enzyme aromatase and block conversion of androgens into estrogens [169].

Aromatase is a cytochrome P450 and is encoded by the CYP19 gene. Aromatase catalyzes the conversions of androstenedione to estrone and of testosterone to estradiol (Fig. 7) and these are the rate-limiting steps in estrogen production [170]. Aromatase is highly expressed in placenta and granulosa cells of the ovary, and lower expression is observed in fat, muscle, subcutaneous, liver and normal breast tissues [171]. Aromatase inhibitors inhibit the action of aromatase, and hence prevent conversion of androgens into estrogens by a process called aromatization [172].

Aromatase inhibitors cannot be used for treating premenopausal women with breast cancers, since 95% of circulating estrogen in premenopausal women is produced in the ovaries, instead of the adrenal gland and inhibition of aromatase does not sufficiently decreases the production of estrogen [173]. In contrast, treatment of premenopausal women with aromatase inhibitors decreases estrogen levels and activates the hypothalamic-pituitary axis to stimulate gonadotropin secretion in the pituitary gland which in turn increases ovarian production of estrogen (Fig. 6).
Figure 7. The role of aromatase in estrogen synthesis.

Aromatase inhibitors can be classified to two types: Type I inhibitors (steroidal) have an androgen structure and interact with the substrate-binding site of the enzyme; Type II inhibitors are azoles (non-steroidal) and associate with the cytochrome P450 heme moiety of the enzyme (Fig. 8) [173]. There are three generations of aromatase inhibitors and these include testolactone (Type I) and aminogluthethimide (Type II) which belong to the first generation; formestane (Type I) and fadrozole (Type II) are second generation aromatase inhibitors; and
exemestane (Type I), anastrozole and letrozole (Type II) belong to the third generation of these compounds [174]. Type I aromatase inhibitors irreversibly inhibit the aromatase activity whereas type II inhibitors reversibly bind to the enzyme in competition with the natural substrates.

The major side-effects of aromatase inhibitors are increased incidence of osteoporosis and bone fracture [175]. In contrast to tamoxifen, aromatase inhibitors are associated with lower incidence of endometrial cancer and thromboembolic phenomena. Aromatase inhibitors are used as second-line medication following tamoxifen treatment and these compounds are effective for treating tamoxifen resistant tumors. In the Intergroup Exemestane Study (IES)
trial, breast cancer patients who were treated with tamoxifen for 2-3 years were assigned to exemestane or continued tamoxifen treatment, and after a 2.5 year follow-up, the exemestane group exhibited a significantly decreased breast cancer recurrence compared to patients treated with tamoxifen [175]. In postmenopausal breast cancer patients who have completed 5 years of tamoxifen treatment, administration of letrozole resulted in a 43% decrease in breast cancer incidence compared to patients using tamoxifen for 2 additional years [176].

1.2.3.3.3 Progestins and antiprogestins

Progestins such as megestrol acetate and medroxyprogesterone acetate are synthetic derivatives of progesterone that have been used for treating metastatic breast cancers in postmenopausal women [177]. The response rates (30%) for progestins are comparable with that of tamoxifen, however they are used as a second-line therapy because of their side-effects which include weight gain and thromboembolic phenomena [178]. It was suggested that antiprogestosterone therapy can also be used to treat breast cancer, since progesterone increases breast epithelium proliferation. Mifepristone (RU486) was the first antiprogestin used clinically, however there was a poor response rate (10.7%). Onapristone, a second generation antiprogestin was successfully used in early clinical trials but was discontinued due to its liver toxicity [179]. New antiprogestins are in development and are more selective for the progesterone receptor [180].
1.2.3.4 Targeted therapy

Although a number of systemic therapies are available for treating breast cancer, most solid tumors develop resistance to systemic agents. Targeted therapies have recently been developed and they are designed to specifically target cancer cells without affecting normal cells. Agents targeting specific molecules in cancer cells are highly selective, have fewer side-effects compared to most traditional chemotherapeutic agents, and can be used in combination with conventional therapies to improve treatment response. Development of targeted drugs for treating breast cancer requires identification of overexpressed or amplified molecules that are important oncogenic pathways in breast cancer.

Growth factor receptors are important for sustaining growth and survival of breast cancer cells, and are important drug targets for treating breast cancer. The epidermal growth factor receptor (EGFR) family of tyrosine kinase receptors plays important roles in the pathogenesis of breast cancer and includes EGFR (ErbB1), ErbB2 (HER2), ErbB3 and ErbB4 receptor kinases. The EGFR is expressed in 14-91% of breast tumors, and overexpression of EGFR in tumors is associated with a more aggressive phenotype and poor prognosis [181]. Expression of ErbB2 occurs in 20-30% breast carcinomas, and ErbB3 and ErbB4 expression is also occurs frequently observed in breast tumors [181].

Ligand-activated ErbB interacts with adaptor proteins that activate intracellular signaling pathways including phosphatidylinositol 3-kinase (PI3K)/v-akt murine thymoma viral oncogene homolog 1 (AKT) pathway and
Ras/Raf/mitogen-activated protein kinase kinase (MEK)/mitogen-activated protein kinase (MAPK) [182]. Activation of these pathways can be ErbB-dependent or ErbB-independent due to involvement of other activated tyrosine kinases.

The PI3K/AKT pathway promotes tumor proliferation, survival, invasion and migration [183]. Dysfunction or loss of expression of phosphatase and tensin homolog (PTEN), the negative regulator of PI3K signaling, and activation of AKT are observed in various cancers [184]. One of the important downstream targets of PI3K/AKT signaling is the serine/threonine kinase mammalian target of rapamycin (mTOR), which activates ribosomal p70S6 kinase (S6K1) and inactivates the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) to regulate translation initiation. RPS6KB1, the gene that encodes S6K1, is amplified in 10% of breast tumors and the amplification is associated with ErbB2 overexpression [185].

The Ras/Raf/MEK/MAPK signaling pathway regulates important events in tumor progression including cell growth and survival, and activation of MAPK signaling has been related to resistance to endocrine therapy and EGFR targeting drugs in breast tumors [186].

The protein kinase C (PKC) is a family of serine/threonine kinase that mediates intracellular signaling [187]. Activation of PKC through phospholipase Cγ (PLCγ) is induced by phosphorylation of EGFR and the vascular endothelial growth factor receptor (VEGFR). Activated PKC activates a series of kinases
including glycogen synthase kinase 3β (GSK3β), which is responsible for development, metabolism and apoptosis. PKC plays a role in breast cancer pathogenesis, and enzymatic activity of PKC is increased in breast carcinomas compared to normal breast tissues [188]. Therefore, PKC is also considered as a target for treating breast cancers.

Src belongs to a large family of nonreceptor protein kinases, and activates various intracellular signaling cascades including PI3K/AKT and Ras/Raf/MAPK pathways. There is evidence that Src plays a role in progression of breast carcinomas and Src kinase activity is 4-20-fold higher in human breast carcinomas compared to normal breast tissues [189]; Src cooperates with EGFR in breast tumor progression [190] and Src increases the metastatic potential of tumor cells through enhancing epithelium to mesenchymal transition [191]. Thus Src is also a good candidate for targeted therapy in breast cancer treatment.

Angiogenesis, namely the formation of new blood vessels, is important for primary tumor growth and metastasis and vascular endothelial growth factor (VEGF) is the major molecule that regulates tumor-related neoangiogenesis. The normal function of VEGF is to produce new blood vessels after injury or during embryonic development, and create collateral circulation to bypass blocked vessels [192]. VEGF binds the vascular endothelial growth factor receptor 1 (VEGFR-1) to activate endothelial cell differentiation and migration; activation of VEGFR-2 by VEGF regulates vascular permeability, endothelial cell survival, proliferation, invasion and migration; VEGFR-3 signaling is associated
with lymphangiogenesis. Overexpression of VEGF is observed in many breast cancers and a correlation between VEGF overexpression and poor prognosis and low response rate to tamoxifen was observed in advanced breast cancer patients [193].

Given the identification of numerous molecular targets in breast cancer therapy, these agents can be classified into three groups: 1) agents targeting ErbB2; 2) anti-angiogenic agents; and 3) inhibitors of specific signaling pathways.

1.2.3.4.1 Anti-ErbB2 agents

Agents that block ErbB receptor family activities have been developed during the past 20 years and these include monoclonal antibodies which bind the extracellular domain of the receptor, and tyrosine kinase inhibitors (TKIs) that inhibit the kinase domain activity of the receptor and some of the key agents are described below.

Trastuzumab (Herceptin) is a monoclonal antibody that blocks the HER2/neu/ErbB2. Trastuzumab binds to the juxtamembrane portion of the extracellular domain of ErbB2 through its two antigen-specific sites. The remaining part of the antibody is the human immunoglobulin G (IgG) with a conserved Fc portion. Mechanisms of action of trastuzumab include interference with ErbB2 homodimerization and heterodimerization with other ErbB members, increased endocytosis and degradation of ErbB2, inhibition of ErbB2 extracellular domain cleavage, and activation of immune responses that kill
tumor cells (Fig. 9) [194]. Immune deficient animals do not respond to trastuzumab, suggesting that trastuzumab recruits immune effector cells and stimulates antibody-dependent cytotoxicity [195]. Studies of ErbB2 overexpressed in animal models of breast cancer suggest that trastuzumab also inhibits angiogenesis, by modulations of proangiogenic and antiangiogenic factors to regress the vascular structure of tumors [196].

Trastuzumab is used for treatment of metastatic breast cancer and adjuvant therapy for early-stage breast cancer. For metastatic and advanced HER2+ breast cancer, trastuzumab is the standard therapy, and treatment with trastuzumab prolongs survival of metastatic breast cancer patients [122]. Trastuzumab is used with or without chemotherapy for first-line treatment of metastatic breast cancer. Use of trastuzumab in adjuvant therapy is considered for women with early-stage HER+ breast cancer. Results from different trials show that adjuvant trastuzumab treatment improves disease-free survival (DFS) and overall survival (OS), but the optimal duration of use is unclear [197].

Cardiotoxicity is one of the major complications of trastuzumab and approximately 7% of patients treated with trastuzumab develop cardiac dysfunction, and regular cardiac exams are taken during treatment with trastuzumab [198]. Clinical oncologists usually need to balance the risk of cardiac dysfunction against cancer recurrence.

Lapatinib is a tyrosine kinase inhibitor (TKI) that binds to the adenosine triphosphate (ATP)-binding site of the intracellular kinase domain of EGFR and
Figure 9. Mechanisms of action of trastuzumab. Trastuzumab blocks cleavage of extracellular domain of ErbB2 (B), inhibits dimerization and hence reduces ErbB2 signaling (C), recruits immune effector cells to activate tumor cell death (D), and enhances ErbB2 endocytosis and degradation (E).
ErbB2. This interaction inhibits the use of ATP as a cofactor for auto-
phosphorylation of the tyrosine residue and inhibits downstream signaling to
activate PI3K/AKT and MAPK pathways [199]. Dissociation of lapatinib from the
intracellular tyrosine kinase domain is very slow (half life≥300 min) and results in
downregulation of EGFR and ErbB2 signaling in tumor cells [200]. Lapatinib
reversibly inhibits EGFR and ErbB2 kinase activities with half maximal inhibitory
centration [IC50] values of 10.2 and 9.7 nmol/L [201], and this inhibition
results in growth arrest and apoptosis in EGFR and ErbB2 positive breast
tumors [202].

Because of its small molecular weight (580 kDa), lapatinib can pass
through the blood-brain barrier and exhibits anti-tumor activity in the central
nervous system (CNS) [203]. A preclinical animal model has shown that
lapatinib inhibits metastases of breast cancer to the brain [204]. Lapatinib is also
a substrate for P-glycoprotein and breast cancer resistance protein (BCRP),
which work together as efflux transporters to regulate penetration of drugs
through the blood-brain barrier.

Lapatinib has been used to treat woman with ER+/EGFR+/HER2+ (triple-
positive) breast cancer and patients with advanced HER2+ breast cancer that
Have not had positive responses with other chemotherapeutic agents.
Resistance to endocrine therapy is a problem for treatment of patients with triple
positive breast tumors and lapatinib re-sensitizes breast cancer cells to
tamoxifen in tamoxifen-resistant breast tumor models [205], and lapatinib also
interacts with antiestrogens to inhibit growth of ErbB2 amplified breast cancer cells [206]. Lapatinib in combination with other chemotherapeutic agents, such as capecitabine, a prodrug of 5Fu significantly decreases progression of ErbB2 overexpressing, trastuzumab-resistant metastatic breast cancer [207].

Pertuzumab is another humanized monoclonal antibody that binds to the extracellular domain of ErbB2. This agent inhibits homo- or hetero- dimerization of ErbB2, and pertuzumab is more efficient than trastuzumab for this activity [208], and is often used in combination with trastuzumab for their complementary action [209]. Frequent adverse side effects of pertuzumab include diarrhea, nausea, pain, skin rash and mucositis. The response rate to pertuzumab is approximately 18%, and the rate of disease stabilization in patients treated with pertuzumab is about 21% [210].

1.2.3.4.2 Anti-angiogenic agents

Anti-angiogenic agents include monoclonal antibody of VEGF (bevacizumab) and TKIs of VEGFRs (sorafenib, sunitinib, candetanib, et al).

Bevacizumab (trade name Avastin) is a humanized monoclonal antibody against VEGF-A, and was the first angiogenesis inhibitor used in the United States. Binding of bevacizumab to VEGF-A blocks the interaction of VEGF-A with VEGFR and hence leads to inhibition of VEGF-related angiogenesis. Bevacizumab was approved by U.S. Food and Drug Administration (FDA) in 2008 to treat metastatic breast cancer, and it was also used as a single agent for treating ovarian cancer, and glioblastoma multiforme. Bevacizumab
demonstrated minimum activity as a single agent for treating metastatic breast cancer; however, combination of bevacizumab with first-line chemotherapeutic agents enhanced their effectiveness in phase III trials. In the E2100 trial, combinations of bevacizimab with paclitaxel improved the progression-free survival and increased the response rate compare to paclitxel alone in patients with metastatic breast cancer [211]. In the avastin and docetaxel (AVADO) trial, addition of bevacizumab to docetaxel significantly prolonged the progression-free survival and response rate compared with docetaxel alone in patients with ErbB2 negative metastatic breast cancer [212].

Other uses of bevacizumab include its application in eye diseases such as macular degeneration (AMD) and diabetic retinopathy, in which VEGF-induced over-growth of blood vessels around the retina causes retina damage and blindness [213]. The major side effect of bevacizumab is hypertension, which is induced or enhanced due to the lack of collateral circulation around blocked vessels and interference with new blood vessels formation in wound healing [214].

Sorafenib is a small molecule that inhibits multiple receptor tyrosine kinases, including VEGFR2, VEGFR3, platelet-derived growth factor receptor (PDGFR)-β, and stem-cell factor receptor (c-KIT). In breast cancer cells with mutated K-Ras and B-Raf, sorafenib also inhibits Raf isoforms including Raf-1 and B-Raf, and decreases activation of MAPK [215]. Sorafenib is primarily used for the treatment of kidney cancer and advanced liver cancer by reducing
angiogenesis. Sorafenib as a single agent for treating metastatic breast cancer exhibited limited activity, and combination of sorafenib with other chemotherapeutic for treating metastatic breast cancer is being investigated [216].

Vandetanib is a TKI that targets VEGFR2, EGFR and rearranged during transfection (RET) receptor tyrosine kinases and minimal effects were observed for this compound in patients with metastatic breast cancer [113]; additional trials on the combination of candetanib and other TKI with chemotherapeutic agents are ongoing. Other anti-angiogenic TKIs include axitinib, which inhibits VEGFR1, 2 and 3 [217]; and pazopanib, which inhibits multiple kinases such as VEGFRs, PDGFR-α/β and c-KIT [218]. Their use in combination with themotherapeutic agents are also being investigated in various clinic trails [219].

1.2.3.4.3 Inhibitors of signaling pathways

Anti-EGFR agents - Gefitinib is an EGFR TKI that extensively inhibits growth of EGFR positive tumors including breast carcinomas. Gefitinib binds to the ATP-binding pocket of EGFR and inhibits kinase activity and the Ras signaling pathway [220]. Gefitinib is extensively used for treating EGFR-overexpressing non-small cell lung cancer (NSCLC) in 46 countries as an all-line treatment that significantly improves the progression-free survival. However, phase II clinical trials of gefitinib as a signal agent demonstrated minimal activity for treating advance breast cancer [221], and effects of gefitinib in combination with chemotherapy is being investigated. Resistance to gefitinib in breast cancer
treatment may be due to loss of dependence on EGFR in certain tumors or EGFR-independent activation of PI3K or MAPK signaling cascades, since inhibition of EGFR phosphorylation by gefitinib has been observed in pharmacodynamic studies [222].

Erlotinib is a reversible TKI for EGFR, and also inhibits ErbB2 kinase activity through a direct interaction with the receptor [223]. Erlotinib was approved by FDA in 2005 for use in treatment of pancreatic cancer in combination with gemcitabine. Treatment with erlotinib in patients with breast cancer prior to surgery dramatically reduced EGFR and ErbB2 kinase activities [224]. Limited activity is observed when erlotinib is used as a single agent or combined with chemotherapeutic agents for treatment of metastatic breast cancer treatment [225].

Cetuximab is a chimeric monoclonal antibody against the extracellular domain of EGFR that decreases EGFR autophosphorylation, endocytosis and degradation. Cetuximab demonstrates anti-tumor activity in a number of tumor xenografts and synergistically interacts with radiation therapy [226]. Cetuximab was approved by FDA in 2006 for treatment of head and neck cancer in combination with radiation. In a phase I trial of cetuximab with paclitaxel in patients with EGFR-overexpressing breast cancer, low activity was observed [227].

mTOR inhibitors - mTOR mutations and overexpression are rarely seen in human tumors, however, mTOR signaling is pivotal for cell growth, proliferation
and survival, and pathways that regulate mTOR are often deregulated in breast cancer. Rapamycin is the first identified mTOR inhibitor identified and temsirolimus and everolimus are two analogues of rapamycin being developed for clinical applications. Temsirolimus, a water-soluble ester derivative of rapamycin exhibits anti-cancer activity in various cancer models. Treatment of breast cancer with temsirolimus leads to G1 arrest and ER+, PTEN negative and / or ErbB2 overexpressing breast cancer cell lines are sensitive to temsirolimus, whereas cell lines lacking of these characteristics are resistant. Temsirolimus also exhibits antiangiogenetic activity [228], and a phase II study in advanced metastatic breast cancer patients showed a 9% response rate after treatment with temsirolimus [229].

Everolimus is another mTOR inhibitor in clinical trials; in cancer cells lacking of PTEN expression, everolimus enhance the growth inhibitory effects of trastuzumab and gefitinib [230]. In 2009 everolimus was approved by the FDA for treating kidney cancer, and clinical trials in breast cancer patients are ongoing.

Farnesyl transferase inhibitors - Farnesyl transferase (FTase) catalyzes addition of a farnesyl isoprenoid to the cysteine residue at the carboxyl terminal of proteins. Targets of FTase include Ras small GTP binding proteins, which are essential for cell cycle progression and are usually highly active in tumor cells. Therefore, FTase inhibitors (FTIs) such as tipifarnib, lonafarnib, and AZD3409 are being developed as anti-cancer agents.
In preclinical studies in ER+ breast tumors, tipifarnib alone and in combination with 4-hydroxytamoxifen showed synergistic anticancer activity in ER+ breast cancers [231]. Lonafarnib exhibits anti-tumor activity in various mouse xenograft models [232], and preclinical studies with combination of lonafarnib and trastuzumab, aromatase inhibitors, or chemotherapy are ongoing. AZD3409 is a novel FTI that mimics the carboxyl terminus CAAX sequence of K-Ras, which is often mutated in human tumors [233]. AZD3409 inhibits both FTase and geranylgeranyl transferase I (GGTI) activities, and also inhibits growth of gefitinib-resistant breast cancer cells [234].

Src inhibitors - Src plays an important role in cell growth, invasion, angiogenesis and metastasis. Inhibition of Src activation can prevent tumor progression and decrease metastasis and Src inhibitors are being developed for treating breast cancers. Dasatinib is a Src kinase inhibitor and is approved for treating chronic myelogenous leukemia (CML). Dasatinib inhibits growth of breast cancer cells and this includes the more aggressive basal-like subtype [235]. Pharmacodynamic studies indicate that phosphorylation of Src is inhibited by dasatinib in dose-dependent manner [236].

AZD0530 is a dual inhibitor that targets the tyrosine kinase domain of Src and Abl. AZD0530 inhibits growth of MCF-7 breast cancer cells and tamoxifen plus AZD0530 exhibits synergistic activity in treating ER+ breast cancer cells, suggesting that inhibition of ER signaling is required for maximal activity of this drug combination [237]. Bosutinib is another dual inhibitor for Src and Abl
kinases that significantly inhibits growth, invasion and migration of MDA-MB-231 breast cancer cell [238], however, side effects of this drug include asthenia, nausea, vomiting, diarrhea, and anorexia.

MEK signaling inhibitors - Many MEK signaling inhibitors are in development, and only AZD6244 (arry-142886) is effective for treating breast cancer. AZD6244 specifically binds to the allosteric inhibitor-binding site of MEK1/2 and stabilizes it in the inactive conformation. \textit{In vivo} studies show that AZD6244 inhibits growth of ZR-75 breast tumors in a mouse xenograft model [239]. Examination of tumor biopsy samples demonstrates that AZD6244 inhibits phosphorylation of MAPK in tumor tissues 1 hour after administration [240]. Side-effects of AZD6244 include diarrhea, nausea, rash, and fatigue and clinical trials with AZD6244 are ongoing.

PKC inhibitors - Enzastaurin is a synthetic bisindolemaleimide that specifically inhibits PKCβ, which in turn regulates VEGF signaling. Blockage of PKC activation by enzastaurin decreases AKT and GSK3β activity, inhibits tumor cell growth, decreases VEGF-mediated angiogenesis, and induces cancer cell death [241]. A phase I study showed that administration of enzastaurin (700 mg daily) is well tolerated, however, a phase II study with metastatic breast cancer patients indicated that treatment with enzastaurin as a single agent was not effective [242]. Breast cancer treatments with combinations of enzastaurin and other chemotherapeutic agents including endocrine therapy are ongoing.
1.2.4 ErbB2 and ErbB receptor family

ErbB2 is an attractive target for treatment of breast cancer due to its overexpression and association with enhanced metastasis and poor prognosis [243]. ErbB2 belongs to the human epidermal growth factor receptor (EGFR, HER) family which includes four structurally related members: ErbB1 (EGFR, HER1), ErbB2 (HER2, neu), ErbB3 (HER3), and ErbB4 (HER4) [244]. The symbol ErbB is given to this protein family due to their homology with the erythroblastic leukemia viral oncogene. These four ErbB family members belong to the receptor tyrosine kinase (RTK) family which includes 20 subgroups, and the ErbB family is called the type I RTK [245]. This RTK subgroup plays important roles in cell survival, adhesion, proliferation, differentiation, and migration.

The ErbB family of proteins contains a cysteine-rich extracellular domain, a transmembrane domain and an intracellular domain. The extracellular domain, also named ectodomain, is highly conserved and upon ligand binding the receptor undergoes a conformational change that results in dimerization and activation of kinase activity [246]. The transmembrane domain is hydrophobic and anchors the receptor across the plasma membrane; the intracellular domain contains a highly conserved C-terminal tail with an ATP-binding pocket for autophosphorylation and for phosphorylation of substrates (Fig. 10) [247].
Figure 10. Structural similarities (A) and differences of ligands (B) for ErbB family RTKs (Adapted from [248]).
These receptors form hetero- or homo-dimers to activate downstream signaling pathways that regulate cell behavior [249]. Since ErbB2 does not have a ligand binding site in the extracellular domain, and ErbB3 does not possess the intracellular kinase activity, therefore these two receptors are not capable of triggering cell signaling as monomers or homodimers [250].

1.2.4.1 EGFR

EGFR is a 170 kDa protein that is encoded by a gene in chromosome 7q12.3-p12.1 [251]. The extracellular domain of EGFR is composed of four domains (I-IV), and domains I and III are for creating the ligand binding pocket. When domains II and IV are juxtaposed, the EGFR receptor is in its “closed” conformation and is unable to bind ligands and dimerize with other receptors; in contrast, when the EGFR is in the “open” conformation, ligands can bind and the EGFR can interact with other receptors to form dimers with exposure of domain II (Fig. 11) [252].

EGFR is important for growth, development, and differentiation of multiple tissues including breast, where it is required for mammary gland development in the late stage of pregnancy [253]. The number of EGFR molecules in normal cells ranges from 40,000 to 100,000, whereas in breast cancer cells the number of EGFR molecules can be $2 \times 10^6$ [254]. EGFR is expressed in approximately 30% of human carcinomas and 50% of glioblastomas and expression is strongly associated with poor prognosis for breast cancer patients [244]. Expression of
Figure 11. Ligand-induced conformational change of extracellular domain of EGFR (Adapted from [255]).
EGFR is particularly high in basal-like breast cancers (54% cases), and the EGFR is a therapeutic target for treating this type of breast cancer [256].

1.2.4.2 ErbB2

ErbB2 is a 185 kDa protein encoded by a gene localized in chromosome 17q21, and was first identified by Schechter and coworkers in 1984 [257]. ErbB2 does not have a ligand binding region in the extracellular domain and there is no endogenous ErbB2 ligand. However, ErbB2 interacts with high affinity with other members of the type I RTK family as a co-receptor due to its extended ligand/heterodimer linkage [258]. A mutation of the ErbB2 gene at amino acid codon 655 is frequently observed in breast carcinomas and this mutation changes the transmembrane conformation to enhance homodimerization of ErbB2 [259]. Homodimerization of ErbB2 caused by this mutation or by ErbB2 overexpression results in increased cancer cell growth, division, and enhances survival [244].

Overexpression of ErbB2 enhances mitosis and survival of breast cancer cells and this is due to activation of the MAPK and PI3K/Akt-mediated pathways [260]; ErbB2 also induces angiogenesis by enhancing VEGF expression [261]. ErbB2-dependent activation of these pathways is due, in part, to overexpression of cyclin-D1, and inhibition of p27\(^{kip1}\), which results in inhibition of cell cycle progression [262].

ErbB2 overexpression is observed in 13%-23% of breast tumors, and is also involved in metastasis and the aggressive phenotype of breast cancer cells.
Overexpression of ErbB2 is linked with increased disease recurrence and poor prognosis for breast cancer patients and this receptor is clearly an important therapeutic target for treatment of breast cancer.

1.2.4.3 ErbB3

The third member of ErbB family, ErbB3, is a 145kDa protein and its gene is localized on chromosome 12q13 [264]. This receptor does not have active tyrosine kinase activity in the intracellular domain; binding of ligands to ErbB3 does not induce phosphorylation and/or receptor activation. However, ErbB3 interacts with other members of ErbB family that harbor the kinase activity to form heterodimers that activate cell proliferation and differentiation [265].

The intracellular tyrosine kinase region of ErbB3 possesses seven Src Homology 2 (SH2) domains that specifically recognize phosphorylated tyrosine residues on other proteins. ErbB3 has high affinity for PI3K, heterodimerizes with other ErbB members and activates survival pathways [266]. ErbB3 is the preferred heterodimeric partner of ErbB2 and overexpression of both receptors are frequently observed; when ErbB2 is inactivated by a neutralizing antibody, decreased ErbB3 signaling activity is also observed [267,268]. One study showed that despite the expression of EGFR and ErbB4, lack of ErbB3 expression strongly decreased the neoplasticity of the breast cancer cell lines, indicating that the ErbB2/ErbB3 heterodimer plays a critical role in tumor cell proliferation and survival [269,270].
Levels of ErbB3 in breast cancer cells are significantly higher than that in normal breast cells, and this is due to enhanced protein translation and/or decreased protein degradation [271]. ErbB3 overexpression is linked to poor prognosis for breast, lung and ovary cancer patients, and approximately 17% of breast cancer patients express high levels of ErbB3 [244].

1.2.4.4 ErbB4

ErbB4, is an 180kDa protein, and is encoded by a gene located in chromosome 2q33 [272]. The cytoplasmic domain of ErbB4 has the capacity to autophosphorylate and does not require other ErbB family receptors to form dimers to activate downstream signaling pathways [273]. There are several alternative splicing isoforms of ErbB4 with similar ligand binding sites and tyrosine kinase activities [273].

ErbB4 is ubiquitously expressed in breast, lung, prostate, ovarian, cervical, stomach and thyroid cancers [273], and ErbB4 levels in breast tumors are lower than EGFR and ErbB2 [274]. ErbB4 is overexpressed in the normal breast during pregnancy and has a function in cell differentiation and lactation [275].

Expression of ErbB4 is associated with good prognosis in a subset of breast cancer patients and they have longer survival times and disease-free intervals. This is partially due to ErbB4-mediated inhibition of ErbB2 activity and the promotion of apoptosis by ErbB2 [276].
1.2.4.5 ErbB ligands in breast cancer

All ErbB members, except for ErbB2, are activated by their specific ligands belonging to the epidermal growth factor (EGF) hormone family which contain an EGF-like domain, and are expressed as ten different genes [182]. Ligands that specifically bind EGFR include EGF, transforming growth factor-alpha (TGF-α), and amphiregulin (AR), and these ligands do not interact with other members of the ErbB family. Heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC) and epiregulin (EPR) are ligands that bind EGFR and ErbB4. Both ErbB3 and ErbB4 interact with neuregulin1 (NRG1) and NRG2, whereas NRG3 and NRG4 only bind ErbB4 (Fig. 10) [277]. Expression of these ligands are elevated in 85% of breast carcinomas [277].

EGF and TGF-α both bind to domains I and III of the ectodomain of EGFR and exhibit 30-40% homology [252]. Expression of EGF is elevated in breast, pancreatic, and liver cancers, and overexpression of this ligand is linked to promotion of cell proliferation [278]. AR interacts only with EGFR and exhibits two different functions, namely to promote proliferation of normal epithelial cell and some cancer cell, or to inhibit growth of certain aggressive carcinoma cells [279].

Expression of HB-EGF is associated with breast cancer cells, however, in breast cancer patients with poor prognosis, HB-EGF level are dramatically decreased [280]. This ligand also inhibits the proapoptotic effects of some anti-cancer drugs [281]. BTC triggers proliferation of breast cancer cells, and
stimulates differentiation of breast epithelial cells to produce neutral lipids [282]. NRGs are expressed in a number of carcinomas, and play an important role in mammary gland development, and NRGs are also highly expressed in invasive breast cancers [283].

1.2.4.6 ErbB dimerization

In the absence of ligand binding, EGFR, ErbB3 and ErbB4 remain in their “tethered” conformation, in which the dimerization arm of the domain II is buried in domain IV [284]. When a ligand contacts one of these receptors, it binds domains I and III to form an extended conformation, in which the dimerization surfaces of domains II and IV are exposed and promote homo- or heterodimerization [285]. ErbB2 does not have an endogenous ligand, and its dimerization arm in domain II is constitutively exposed and readily heterodimerizes with other ligand-bound ErbBs [286]. Upon ligand binding in the ectodomain, the kinase region located on the tail of these receptors is activated. The kinase region is composed of C and N lobes that interact to achieve the active conformation. Activation of the kinase requires the C lobe in the first receptor to contact the N lobe of the second receptor (Fig. 12) [287], and this results in activation of downstream pathways [288].

Kinase domain mutations of ErbB2 are observed in cancer cells, and this results in constitutive activation of the receptor [289]. Heterodimerization such as EGFR/ErbB2, EGFR/ErbB3, and ErbB2/ErbB3 occur frequently in aggressive carcinomas [290], and the EGFR/ErbB2 combination is observed in the most
aggressive tumors and these patients have a poor prognosis and decreased survival [291].

1.2.4.7 Endocytosis and recycling of ErbBs

Normally, growth factor receptor-coupled signaling is rapidly attenuated by mechanisms that include dissociation of the ligand-receptor complex, dephosphorylation of the activated receptor, rapid internalization through clathrin-coated pits and degradation of active receptors in lysosomes. For ErbB family members, EGFR is the most well studied receptor regarding endocytosis. Ligand-bound EGFR is rapidly endocytosed to form early endosomes, and in the late endosomes the ligand is dissociated from the receptor and undergoes lysosomal degradation; the receptor can also be degraded or recycled back to the cell surface.
EGFR homodimers are usually degraded by lysosomes, however, ErbB2-containing heterodimers are transported back to the cell surface and activated. There are several possible mechanisms that ErbB2-containing heterodimers avoid efficient endocytotic degradation. For example, the rate of EGF-induced internalization of a chimeric receptor consisting of the extracellular domain of EGFR and intracellular domain of ErbB2 was decreased by three- to four-fold compared to wild type EGFR, and the lower internalization rate of the chimeric receptor resulted in lower rates of receptor degradation [292]. It was also reported that geldanamycin-induced cleavage of ErbB2 within the cytoplasmic domain produces a 135-kDa and 23-kDa fragments, with the former representing the extracellular domain and part of the cytoplasmic domain, and the latter the carboxyl-terminal domain; Cleavage of the C-terminal domain resulted in accelerated endocytosis and degradation of the 135-kDa fragment of ErbB2 [293]. Both studies suggest that the C-terminal domain of ErbB2 expresses an inhibitory signal for clathrin-coated endocytosis of the receptor. c-Cbl protein is an E3 ubiquitin ligase that recognizes tyrosine-phosphorylated substrates through the SH2 domain and controls the recycling and degradation of proteins [294]. A number of reports have shown that upon activation of EGFR, c-Cbl is associated with the phosphorylated Tyr-1045 of EGFR in the early endosome, ubiquitinates the receptor and directs it to the lysosome; c-Cbl mediated ubiquitination is required for EGFR endocytosis and lysosomal interactions [295-300]. In contrast, interactions between c-Cbl and ErbB2 are
weak and ubiquitination of ErbB2 followed by activation of the receptor is barely observed; ErbB3 and ErbB4 do not have any affinity for c-Cbl [301,302].

At present, very little is known regarding the endocytosis of ErbB3 and ErbB4, and results of the first study indicate that internalization of these receptors is impaired [303].

1.2.4.8 Regulation of ErbB2 expression

ErbB2 overexpression in breast cancer cells is due not only to impaired degradation, and gene amplification, but also to elevated gene expression. The human ErbB2 proximal promoter consists of typical TATA box (-22 to -26bp) and a CCAAT box (-71 to -75bp) (Fig. 13) and there are two transcription initiation regions at +1, and -69 (Fig. 13). Transcription initiation at these two sites utilizes two separate mechanisms, with the upstream initiation site specified as an initiator-like element, whereas the downstream sites require the TATA box.

![Figure 13. Cis elements and their corresponding trans-acting factors on the ErbB2 promoter.](image)

In ErbB2 overexpressing cells, the upstream initiation site is preferentially upregulated [304,305]. A number of transcription factors bind the ErbB2 proximal promoter, and two of these, activating protein 2 (AP-2) and E-twenty six (Ets) family of transcription factors are required for maximal promoter activity. The two
transcription factors are associated with overexpression of the gene in breast cancers.

AP-2 is a member of a family of closely related transcription factors that regulate gene expression during early development [306-308]. There are two AP-2 binding sites (GCTGCAGGC) at -215 and -500 that are activated in ErbB2 overexpressing cells [309,310], and mutations that block AP-2 binding result in decreased activity of promoter-reporter constructs in ErbB2 overexpressing cells [311]. AP-2 family proteins include AP-2α, AP-2β, and AP-2γ, all of which can activate the ErbB2 promoter [312], and levels of AP-2α, AP-2β, and AP-2γ proteins correlate with ErbB2 overexpression in tumor derived cell lines and primary breast tumors [312,313].

Ets family transcription factors contain 12 subgroups and these protein all contain an ETS domain, which is a conserved helix-turn-helix DNA binding structure that recognizes the “GGA” DNA sequence. The Ets binding site (EBS; GAGGAA) in the ErbB2 promoter is at -33 to -28, and mutation of these sites also impairs activity of ErbB2 promoter-reporter constructs [305,314]. More than 10 different Ets proteins have been identified in breast cells, but only PEA3 expression correlates with ErbB2 overexpression [315]. In addition, there are conflicting reports regarding the effects of PEA3 on the ErbB2 promoter. One report showed that PEA3 and ErbB2 are coordinately upregulated in human breast tumors and PEA3 enhanced ErbB2 promoter activity in COS cells [315]; However, Xing et al observed that PEA3 represses ErbB2 expression through a
positive regulatory motif, PEA3 suppresses growth of MDA-MB-453 breast cancer and SKOV3 ovarian cancer cells that overexpress ErbB2, and inhibits growth of ErbB2 overexpressing tumors in vivo [316].

Ying Yang 1(YY1) is another transcription factor that also plays a role in ErbB2 expression. YY1 regulates the expression of a number of genes [317], and this protein is composed of an activation domain, two repression domains and a DNA binding domain. YY1 can function as a transcriptional activator or repressor [318], and YY1 expression correlates with ErbB2 and AP-2 overexpression in primary breast tumors [319,320]. Full length but not a C-terminal truncated YY1 enhances AP-2-mediated transactivation through an AP-2 site in the ErbB2 promoter and inhibition of endogenous YY1 expression in BT474 breast cancer cells decreases AP-2 responsive-promoter activity. Interactions of endogenous AP-2 and YY1 and their association with ErbB2 promoter have been reported, suggesting that YY1 cooperates with AP-2 to activate ErbB2 transcription in breast cancer cells [321].

MicroRNAs (miRs) that target the 3’ untranslated region (3’ UTR) of ErbB2 and ErbB3 mRNAs include miR-125a and miR-125b and these two miRs are significantly downregulated in ErbB2 overexpressing breast cancers [322]. Overexpression of miR125a and miR125b in SKBR3 breast cancer cells in which ErbB2 is overexpressed decreases ErbB2 and ErbB3 transcripts and protein levels [323].
1.2.4.9 Major ErbB2 signalings

In ErbB2-overexpressing cancer cells, heterodimers between ErbB2 and other members of the ErbB family are predominant and the most well-characterized ErbB2-induced signaling involves activation of Ras/MAPK, PI3K/Akt, and PLCγ/PKC pathways (Fig. 14). Ligand induced ErbB dimerization induces autophosphorylation of the intracellular kinase domain, which in turn phosphorylates Src homology 2-containing protooncogene (Shc). Phosphorylated Shc recruits adaptor protein, growth factor receptor bound 2 (Grb2), and stimulates activation of Son of Sevenless (Sos) guanine nucleotide exchange factor. Activated Sos removes guanosine diphosphate (GDP) from Ras and adds guanosine-5'-triphosphate (GTP). GTP-bound Ras associates with and activates the serine/threonine kinase Raf, which in turn phosphorylates MEK1, and activates p42/p44 MAPK (also known as extracellular signal-related kinase 1 and 2 (ERK1 and ERK2)). Activated MAPK phosphorylates cytoplasmic and nuclear substrates including other protein kinases, growth factor receptors and transcription factors that are involved in proliferation, differentiation and cell survival [324].

Activation of the PI3K pathway through ErbB receptors requires the p85 regulatory subunit of PI3K, which contains an SH2 domain that recognizes and binds phosphorylated tyrosines that are autophosphorylated by the kinase activity of the receptor. The activated p110 catalytic subunit of PI3K phosphorylates phosphatidylinositol (PI) to produce phosphatidylinositol 4,5-bis-
Figure 14. Major signaling pathways of ErbB2 (Adapted from [325]).
phosphate(PIP$_2$) and phosphatidylinositol (3,4,5)-trisphosphate(PIP$_3$). These PI-phosphates function as docking sites for pleckstrin homology (PH) domain-containing proteins such as phosphatidylinositol dependent kinase-1(PDK1) and PKB/Akt which in turn transmit signals from the cell membrane into the cell. Akt/PKB is phosphorylated and activated by PDK1 and released into the cytosol, where it phosphorylates target proteins and regulates cell proliferation, metabolism, and apoptosis. ErbB2 and EGFR do not have binding sites for PI3K, however, active ErbB3 contains six PI3K binding sites; therefore ErbB2/ErbB3 and EGFR/ErbB3 heterodimers can generate potent signals through the PI3K pathway.

Activation of PLC$_{\gamma}$ by ErbB2 is initiated by recognition of the phosphotyrosine sites by the SH2 domain of PLC$_{\gamma}$, then the activated PLC$_{\gamma}$ is recruited to the plasma membrane through the binding of its PH-domain to phosphatidylinositol lipids within the membrane where PLC$_{\gamma}$ hydrolyses PIP2 to IP3 and diacylglycerol. This reaction results in activation of PKC, calcium/camodulin-dependent kinases and phosphatases, which are involved in development, metabolism and apoptosis [326].
1.3 Sp transcription factors as drug targets

1.3.1 Introduction of Sp / KLFs

Specificity proteins (Sps) are highly related zinc finger proteins that play important roles in eukaryotic cell transcription through regulating expression of a number of genes that contain GC-rich promoters. Sp1 was the first transcription factor identified and was characterized in 1980 [327,328]. Other transcription factors that are similar to Sp1 were also identified as Sp1-like proteins and Krüppel-like factors (KLFs) [329]. At least 21 Sp/KLF human genes have been identified using different cloning approaches. The Sp/KLF family is subdivided into several subgroups based on their structural similarities and Sp1-Sp6 form subgroup I in which all members are highly related to Sp1[330].

1.3.1.1 Structural features

A typical transcription factor contains a DNA-binding domain, a nuclear localization signal, and a transcriptional regulatory domain. The DNA-binding domain of Sp/KLF proteins is highly conserved and contains three tandem Cys$_2$His$_2$ zinc-finger motifs that are localized at the carboxyl terminus. The similarity between zinc finger motifs of Sp1 and other Sp/KLF genes is 66.7%, but there are differences in DNA binding specificity. For example, Sp2 has a leucine residue in the first zinc-finger motif, whereas Sp1 has a histidine residue in the same region and Sp2 preferentially binds GT-rich (5’-GGTGTGGGG-3’) rather than GC-rich sites which are bound by other Sp/KLF proteins [331,332]. A conserved nuclear localization signal adjacent to or within the zinc-finger motifs
is observed only in KLF1, KLF2 and KLF4 proteins which belong to subgroup II of the Sp/KLF protein family [333,334]. The transcriptional regulatory domains of Sp/KLF proteins are located in the amino-terminus and are highly variable in their inhibitory or activation functions. Sp1, Sp3 and Sp4 have two glutamine-rich domains A and B in their respective transcriptional regulatory domains and are essential for activation of transcription, whereas Sp2 has only one glutamine-rich domain. Serine/threonine rich sequences are located adjacent to the A and B domains. The buttonhead (Btd) box is a conserved sequence of 11 amino acid located in the N-terminal of the zinc-finger domain of all Sp proteins, and is essential for the their transactivation potential [335]. Another region of conserved amino acids (sequence of SPLALLAATCSR/KI) has been identified, and this element (Sp-box) contains an endoproteolytic cleavage site reside in the N-terminus of these proteins that targets proteasome-dependent degradation [336] (Fig. 15). It is worth noting that Sp3 has a repression domain located at the 5’ region of the zinc-finger domain, and this inhibitory domain (ID) may function by interacting with other corepressor proteins such as Sp3 interacting protein (SIF-1) [331].
1.3.1.2 Distribution and functional properties of Sp1, Sp3 and Sp4 proteins

Sp1- Sp1 is ubiquitously expressed and plays important roles in embryogenesis, development, proliferation and apoptosis [338,339]. Sp1 regulates transcription of TATA-containing and TATA-less target genes through both proximal and distal enhancers [340]. Sp1 activates transcription synergistically by forming homomultimeric complexes such as tetramers, and the activation domain B appears to be critical for the multimerization [341]; synergistic activation of promoters by Sp1 through binding multiple GC-boxes requires the C-terminal domain D[341]. Sp1 interacts with different classes of nuclear proteins including general transcription machinery factors (GTFs) such as TATA-box binding protein (TBP) and TBP-associated factors TAFII110/TAFII130 and TAFII55 to stabilize the transcription initiation complex [342-344]. In addition, cofactors required for Sp1 activation (CRSP) complex are
required for Sp1-mediated transactivation. Other transcription factors that interacts with Sp1 to modulate transcription include GATA1, E2F, YY1, c-Jun, p53, pRb and many other nuclear factors [345]. Sp1 regulates transcription of large number of genes, such as housekeeping, tissue-specific and cell cycle regulated genes.

The DNA binding ability, transactivational activity, and protein stability of Sp1 can be influenced by posttranslational modifications such as phosphorylation, glycosylation, ubiquitination, acetylation and sumoylation [345]. For example, phosphorylation at Thr453 and Thr739 of Sp1 by p42/p44 MAPK significantly increased transcriptional activity of Sp1 in activation of the VEGF promoter [346]; inhibition of Sp1 phosphorylation at Thr278/739 via inhibition of c-Jun NH2-terminal kinase 1 (JNK1) activity resulted in ubiquitination and degradation of Sp1 [347]. Sp1 glycosylation is through the O-linkage of the monosaccharide and N-acetylglucosamine and Sp1 acetylation are involved in self association, interaction with GTFs and proteasome-dependent degradation [348,349].

Sp3-Sp3 is ubiquitously expressed and Sp3 null mice exhibited abnormal tooth formation and growth retardation although the specific physiological functions are not well defined [350]. In some studies, Sp3, like Sp1 acts as a transcriptional activator [351,352], whereas Sp3 can also be a weak activator or a repressor [353,354]. The structure of promoters is important for determining the function of Sp3. Promoters with a single GC-rich Sp binding site are
activated by Sp3, whereas promoters with multiple Sp binding sites are minimally responsive to Sp3 [355,356]. Cell context is another important factor that determines the function of Sp3; For example, Sp3 transactivates HERV-H long terminal repeat in teratocarcinoma cells but acts as a repressor in Hela and insect cells [357]. Since the Sp1/Sp3 ratio is relatively high in endothelial cells and low in non-endothelial cells, it has also been suggested that the relative abundance of Sp1 and Sp3 in a specific cell line influences the transcriptional activity of Sp3. There are three isoforms of Sp3 which are transcribed from the same gene at different start sites [332]; another study suggests that the two small Sp3 isoforms act as repressors whereas the full length Sp3 isoform acts as an activator [358]. The molecular basis for the suppressive activity of Sp3 is due to the amino acid triplet KEE in the inhibitory domain (ID). Mutation of these amino acids to alanines changes Sp3 into a strong activator, suggesting that the KEE sequence is essential for the suppressive activity of Sp3 [356]. Interactions of Sp3 with corepressors through the ID domain may also contribute the activity of Sp3 as a transcriptional repressor [331]. Posttranslational modifications also regulate the repressor activity of Sp3. For example, modification of Sp3 by small ubiquitin-related modifier-1 (SUMO-1) converted Sp3 into a strong activator of transcription [359].

Sp4-Sp4 is a tissue specific member of the Sp-family the predominant distribution in the brain and also expression in epithelial tissues, testis and developing teeth [360,361]. Sp4 plays a role in postnatal survival and male
fertility [361]. Approximately two-thirds of the Sp4-/- mice died shortly after birth, and the male survivors exhibited defects in copulation and significant growth retardation is observed in both males and females. Like Sp1, Sp4 exhibits similar transactivation potential through the glutamine-rich activation domains. However, in contrast to Sp1, Sp4 does not act synergistically through interactions with Sp proteins bound to adjacent sites [362].

1.3.2 Sp proteins as drug targets

Sp1-Sp4 regulate expression of multiple genes in normal tissues and in tumors. There is growing evidence indicating that some Sp proteins play important roles in growth and metastasis of a number of tumor types and this will be discussed in the following subsection.

1.3.2.1 Overexpression of Sp proteins in tumors

Sp1 is overexpressed in gastric tumors and is non-detectable in stromal or surrounding normal glandular cells [363]. Sp1 is also highly expressed in pancreatic tumors compared to normal tissues, and this overexpression is correlated with increased expression of VEGF which is an Sp-regulated gene [364]. Levels of Sp1 protein are elevated in 11 out of 14 breast carcinomas, and is only detectable in 1 out of 5 benign breast tumors [365], and Sp1 is also overexpressed in thyroid tumors but not in the normal thyroid [366]. Expression of DNA-dependent protein kinases Ku70 and Ku80 is regulated through Sp1 interactions with GC-rich regions in their respective promoters, and theses
kinases are upregulated in colon tumors compared to surrounding normal tissues and this upregulation is correlated with elevated Sp1 expression [367].

Wang and coworkers showed that gastric tumor patients with high Sp1 expression have lower rates of survival than patients with low Sp1 levels [363], and correlation of VEGF and Sp1 overexpression in gastric tumors is also associated with decreased survival times [368].

1.3.2.2 Regulation of growth promotion and cell survival genes by Sp in cancer cells

The role of Sp1, Sp3 and Sp4 in Panc-1 pancreatic cancer cells was investigated using RNA interference (RNAi) and knockdown of Sp1 protein in Panc-1 cells decreased the percentage of cells in G2/M and S phase by 5.52% and 5.74%, respectively, and increased the percentage in G0/G1 by 10.26% [369]. However, when transfected with small inhibitory RNA for Sp3, the percentage of Panc-1 cells in G0/G1 was increased (20.14%) and the percentage in S phase was decreased (17.39%). It was subsequently shown that knockdown of Sp3 by RNAi in Panc-1 and other pancreatic cancer cell lines resulted in upregulation of the cyclin-dependent kinase inhibitor p27 [369]. These results indicated that Sp3 promotes pancreatic cancer cell growth through interaction with the GC-rich region on the p27 promoter to inhibit expression of this gene.

Loss of growth inhibitory pathways is critical for oncogenesis in some tumors, and Sp proteins also play a role in tumorigenesis by Sp3-dependent
suppression of transforming growth factor β receptor (TGFβR) pathway [370-372]. The TGFβRII promoter contains GC-rich elements and Sp1 and Sp3 are required for regulation of expression of this gene [370,371,373]. Late passage MCF-7 breast cancer cells (MCF-7L) are nonresponsive to TGFβ due to nondetectable expression of TGFβRI and TGFβRII, and the Sp1/Sp3 ratio in this cell line is lower than that in TGFβ-responsive early passage MCF-7 cells (MCF-7E) in which TGFβR is expressed [372]. Therefore, Sp3 acts as a repressor for TGFβRII and blocks an important growth inhibitory pathway in breast cancer cells.

Sp1 also regulates a number of genes in breast cancer cells that are involved in cell cycle progression and cell growth (cyclin D1, E2F1, c-fos, TGFα) [374-377], purine/pyrimidine synthesis and metabolism (thymidylate synthase, adenosine deaminase, DNA-polymerase α, carbamylphosphate synthase/aspartate carbamyltransferase/dihydroorotase (CAD)) [378-381], angiogenesis (VEGF) [369,382], and survival (bcl-2, survivin) [383-385].

1.3.2.3 Strategies for targeting Sp transcription factors in cancer cells

Sp transcription factors are highly expressed in cancer cells, and are negative prognostic factors for patient survival and regulate expression of growth promoting, survival and angiogenic / metastatic genes. Approaches for targeting Sp-regulated genes and pathways include chemicals that inactivate GC-rich DNA elements, oligonucleotides and DNA-peptide nucleic acid chimeras that decoy the Sp1 DNA-binding domain, and compounds that regulate Sp protein
expression [386]. For example, treatment of cells with mithramycin, a compound that binds GC-rich motifs decreased Sp1-DNA binding and expression of Sp-regulated genes [387-389]. Hedamycin also binds G-rich DNA sequences and this drug inhibits transcription of survivin through interacting the GC-rich Sp1 site on the survivin promoter [390]. Double stranded oligonucleotides with multiple CG-rich sequences were developed as Sp1 decoys, and transfection of Sp1 decoys into human lung adenocarcinoma and glioblastoma cancer cell lines inhibited Sp1-dependent expression of VEGF and TGFβ1 [391]. Various decoys for Sp transcription factors and other transcription factors are being developed and there may be clinical application of this approach for treatment of different cancers [392,393]. Compounds that modulate Sp protein expression will be described in the following subsection.

1.3.3 Drugs that target Sp downregulation

1.3.3.1 NSAIDs (celecoxib, tolfenamic acid, aspirin)

Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit synthesis of prostaglandins through inhibiting cyclooxygenase (COX)-mediated inflammatory responses, and these drugs are used primarily to treat inflammation, pain, and fever, with specific uses including the treatment of migraines, arthritis, sports injuries, and menstrual cramps. Recent studies showed that COX-2 is overexpressed in numbers of tumor types and promotes tumorigenesis via enhancement of cancer cell proliferation, increased tumor angiogenesis, decreased tumor cell apoptosis, and enhanced tumor metastasis. NSAID-
induced anticarcinogenic activities are due to inhibition of these processes in a COX-2-dependent or independent manner [394-396]. The potential use of NSAIDs in reducing cancer risk is supported by epidemiologic studies which demonstrate that low-dose and regular use of NSAIDs reduced risk of colon cancer [397]. NSAIDs are also effective for preventing tumorigenesis of lung, breast, prostate, pancreatic and esophageal cancers [398-401]. NSAIDs can be classified based on their chemical structures and these include propionic acid derivatives, acetic acid derivatives, enolic acid (oxicam) derivatives, fenamic acid derivatives; according to the function, some of these drugs can be grouped into selective COX-2 inhibitors, and non-selective COX inhibitors which inhibit both COX-1 and COX-2.

Celecoxib is a selective COX-2 inhibitor that is used for treatment of pain and inflammation, and this compound also decreases the numbers of colon and rectum polyps in patients with familial adenomatous polyposis. A number of studies indicate that celecoxib can be used for cancer prevention and treatment. Epidemiologic studies showed that people taking NSAIDs such as aspirin or celecoxib on a daily basis have a lower risk of colon cancer; carcinogenesis studies in rodents show that prevents formation of intestinal cancers [402]; small scale clinical trials in individuals with familial adenomatous polyposis show that celecoxib prevents polyp growth, and large scale clinical trials show 33 to 45% reduction of polyp growth in people taking celecoxib regularly [403]. Celecoxib inhibits COX-2 and also modulates other intracellular pathways, and several
celecoxib analogs devoid of COX-2 inhibitory activity also exhibit anticancer activity, indicating that COX-2 inhibition is not required for the anticancer effects of celecoxib [404,405]. In addition, celecoxib decreases Sp1 and VEGF expression and through induction of Sp1 degradation in pancreatic cancer cells [406], and COX-2 inhibitors inhibit VEGF expression by decreasing Sp1 and Sp3 levels in colon cancer cells [407].

Tolfenamic acid belongs to the fenamic acid subgroup of NSAIDs, and has been used to treat migraines [408-412]. It was also reported that tolfenamic acid inhibited pancreatic cancer cell and tumor growth by targeting downregulation of Sp1, Sp3 and Sp4 proteins [413]. In this study, tolfenamic acid was the most potent among 14 screened NSAIDs (COX-1 and/or COX-2 inhibitors) for the ability to decrease Sp1, Sp3 and Sp4 protein expression in pancreatic cancer cells through the proteasome pathway. Tolfenamic acid also inhibited VEGF mRNA and protein expression in pancreatic cancer cells, and this inhibition was related to decreased Sp-dependent activation of the VEGF promoter.

Aspirin (also known as acetylsalicylic acid, ASA) was the first NSAID discovered, and is one of the most widely used medications in the world. Approximately 40,000 tones of ASA being consumed each year as an antipyretic to reduce fever, as an anti-inflammatory medication, and for prevention of heart attacks, strokes, and formation of blood clots [414,415]. There is growing evidence showing the association of between aspirin use and the reduced
incidence in colon, breast, lung, stomach, oesophagus, bladder, ovary, prostate, mouth, and skin cancers [416,417]. Aspirin decreases cancer risk through inhibition of COX. Salicylic acid which is the main metabolite of aspirin can suppress COX-2 mRNA and protein expression in human endothelial cells and fibroblasts [418], and aspirin decreases COX-2-dependent synthesis of prostaglandins which enhance carcinogenesis through promotion of cell survival, cell proliferation and angiogenesis [419-421]. The acetyl group of aspirin trans-acetylates serine 530 within the active site of COX-1, and inhibits COX-1 mediated platelet aggregation, vascular homoeostasis, renal blood flow, and glomerular function. Acetylation of the COX-1 active site blocks the access of the COX-1 substrate arachidonic acid to the active site; in contrast the enzyme active site of COX-2 is larger than that of COX-1, and is accessible to acetylated substrates [422]. A second possible mechanism for the anticancer activity of aspirin is due to induction of apoptosis. Aspirin induces apoptosis by activation of caspases [423], activation of p38 MAP kinase [424], activation of ceramide pathway [425], release of mitochondrial cytochrome c [426], induction of a proapoptotic gene PAWR [427], and downregulation of antiapoptotic genes such as BCL2 [428]. These effects may not be observed in all cell types and the doses of aspirin required for these COX-independent pathways are higher than doses that inhibit COX-2. Aspirin also inhibits NFκβ, which is a transcription factor that activates anti-apoptotic genes, and this effect contributes to the decreased incidence of colon, breast and gynaecological cancers associated
with aspirin use [429]. Moreover, aspirin can upregulate tumor suppressor genes such as p53, p21, and BAX [428]. DNA mismatch repair protects against the accumulation of mutations during DNA-replication and it was also reported that aspirin decreases microsatellite instability in mismatch-repair deficient colon cancer cells [430]. In another study, aspirin increased levels of mismatch-repair proteins and inhibited growth of mismatch-repair-deficient colon cancer cell lines [431]. Other possible mechanisms of the anticancer activity of aspirin include inhibition of mitochondrial calcium uptake, which is an ubiquitous influx pathway for cell proliferation [432]; decrease in DNA damage through modulation of oxidative stress and glutathione peroxidase activity [433]; inhibition of phenolsulphotransferase, which can activates some carcinogens activator [434]; and prostaglandin-independent effects on angiogenesis [435].

1.3.3.2 Phytochemicals (betulinic acid, curcumin)

Phytochemicals are natural biochemicals produced by plants for protection from insects, disease organisms, oxidation and other environmental hazards. Some phytochemicals have been traditionally used for treatment or prevention of cancer. Examples of cancer-suppressing phytochemicals include curcumin, β-carotene, epigallocatechin gallate, genistein, resveratrol, gingerol, and capsaicin. Most of these cancer-suppressing phytochemicals act on signaling pathways that have been abnormally activated or silenced in tumors and phytochemicals that suppress cancer through repression of Sp proteins will be described in this subsection.
Betulinic acid (BA) is a natural pentacyclic triterpenoid extracted from the bark of several species of plants, principally the white birch (*Betula pubescens*) from which it gets the name. BA has anti-retroviral, anti-malarial, anti-inflammatory, and anticancer properties. BA was initially reported as a potent antimelanoma compound in 1995, and subsequently, it was shown to be effective against cancer cells from other tumor types, including neuroectodermal tumor cells, malignant brain tumors, ovarian carcinomas, nonsmall-cell and small-cell lung carcinomas, cervix carcinomas [436,437]. Normal cells such as melanocytes were much less sensitive to the growth inhibitory effects of BA compared to melanoma cells [438]; BA induced apoptosis in an immortalized human keratinocyte cell line HaCaT, whereas BA induced differentiation of normal keratinocytes into corneocytes [439]; moreover, human skin fibroblasts and peripheral blood lymphocytes are highly resistant to BA [440]. The underlying molecular mechanisms required for the differential effect of BA on cancer versus normal cells are unclear. The anti-apoptotic effects of BA have been extensively investigated, and unlike conventional anticancer drugs which induce apoptosis through activation of death receptor pathways or activation of p53 and subsequent Bax/Bak-dependent release of cytochrome c from mitochondria, BA directly induces of mitochondrial damage resulting in Bax/Bak-independent release of cytochrome c [441-443]. About 50% of human tumors acquire p53 mutations and most of the remaining tumors exhibit p53-deficient pathways, therefore anticancer therapies that are dependent on p53 activation
are prone to become drug resistant, and since BA directly targets mitochondria to induce apoptosis, this drug has great promise for overcoming drug resistance in tumor cells [443-445]. Treatment with BA also increases mitochondrial membrane permeabilization through generation of reactive oxygen species (ROS), and pretreatment with antioxidants can rescue cells from BA-induced apoptosis suggesting that ROS production is important for cell death [437,446,447]. BA can also activate proteasomes. One study demonstrated that the anticancer effects of BA were due in part to degradation of Sp1, Sp3 and Sp4 transcription factors. The protein synthesis inhibitor cycloheximide had no effect on BA-induced downregulation of Sp1, Sp3 and Sp4 protein levels in prostate cancer cells, however cotreatment with the proteasome inhibitor MG132 reversed the BA-induced repression of Sp proteins, suggesting that BA induce proteasome-dependent degradation of Sp proteins [448]. Another study showed that BA directly interacted with purified proteasomes and activated chymotrypsin-like proteasome activity, whereas derivatives of BA with modifications at C-3 exhibited proteasome-inhibitory effects [449]. Other targets of BA include NF-κB, topoisomerases, aminopeptidase N, acetyl-CoA acyltransferase and diacylglycerol acyltransferase, and cell cycle related genes [450-459]. In vivo studies of BA showed that a dose of 50 mg/kg every 4 days administrated intraperitoneally was enough to prevent tumor outgrowth and six injections resulted in tumor regression [460]. In a prostate cancer murine xenograft model treatment with 10 or 20 mg/kg BA administrated orally every
other day significantly inhibited tumor outgrowth compared to a corn oil-treated control [448], suggesting that BA is active even after oral application. Similar activity is observed when BA is applied by intravenous injection in a human murine xenograft model [461], and systemic signs of toxicity were not observed in these in vivo studies.

Curcumin (diferuloylmethane) is the active component of turmeric, and is a polyphenolic natural product that is widely used in cooking and as a traditional medicine by people from southeast Asia [462-464]. The anticancer effects of curcumin have been extensively investigated in various cancer cells and animal models, and the effects are highly variable and dependent on tumor type and cell context. Curcumin induces apoptosis, inhibits cancer cell proliferation, and angiogenesis [465-474]. Curcumin inhibited growth-induced apoptosis in KU7 and 253JB-V bladder cancer cells and this was accompanied by decreased Sp protein expression [475]. Curcumin induced proteasome-dependent downregulation of Sp1, Sp3 and Sp4, and curcumin-induced inhibition of NFκB-dependent genes such as bcl-2, survivin and cyclinD1 is also partially dependent on downregulation of Sp proteins. Curcumin also decreases growth of bladder tumor in KU7 cell mouse xenografts and this was also accompanied by decrease in Sp1, Sp3 and Sp4 protein levels in tumors. Subsequent studies showed that downregulation of Sp1, Sp3 and Sp4 by curcumin in bladder cancer cells is accompanied by decreased EGFR mRNA and protein levels, and it was shown by RNA interference studies that EGFR is an Sp-regulated gene [476].
Another study showed that curcumin decreased expression of Sp proteins and Sp-dependent proteins in Panc28 and L3.6pL pancreatic cancer cells and xenograft mouse models [477]. Moreover, RNAi experiment showed that p65 and p50 subunits of NFκB are regulated by Sp proteins and inhibition of NFκB expression by curcumin is also partially due to loss of Sp transcription factors. Curcumin also decreases mitochondrial membrane potential and induces reactive oxygen species (ROS), and this was essential for downregulation of Sp proteins in pancreatic cancer cells [477].

1.4 Research Objectives

1.4.1 Objective 1: Mechanisms of TA action in breast cancer cells

Tolfenamic acid (TA) is a non-steroidal anti-inflammatory drug (NSAID) that inhibits pancreatic cancer cell and tumor growth through induction of proteasome dependent degradation of specificity protein (Sp) transcription factors [413]. In this research, it was observed that TA also inhibited growth of ErbB2-overexpressing BT474 and SKBR3 breast cancer cells. In contrast to pancreatic cancer cells, TA downregulates ErB2 but not Sp proteins. Therefore, the first objective of this research was to investigate the mechanisms of anti-cancer activity of TA in BT474 and SKBR3 breast cancer cells through downregulation of ErbB2. TA-induced inhibition of ErbB2 gene expression will be determined using real-time PCR, and by transfecting the breast cancer cells with a construct containing the ErbB2 promoter linked to a luciferase reporter gene; the levels of two ErbB2 transcriptional activators AP2 and YY1 will be
determined by western blot analysis; ErbB2 RNA stability will be measured using actinomycin D in combination with TA, and mRNA levels will be determined by real-time PCR. Proteasome dependent degradation of ErbB2 will be investigated using lactacystin, a proteasome inhibitor, in combination with TA. Endocytosis of ErbB2 induced by TA will be determined by immunofluorescence microscopy. Effects of TA \textit{in vivo} will use an athymic nude mouse xenograft model.

\textbf{1.4.2 Objective 2: Mechanisms of BA action in breast cancer cells}

Betulinic acid (BA) has been reported to inhibit prostate cancer cell growth through induction of proteasome dependent degradation of Sp1, Sp3 and Sp4 transcription factors \cite{448}. BA also inhibits growth of ErbB2-overexpressing BT474 and MDA-MB-453 breast cancer cells; Both Sp proteins and ErbB2 are downregulated in BA treated breast cancer cells. The objective of this research will be to investigate the mechanisms of anti-cancer activity of BA in BT474 and MDA-MB-453 breast cancer cells through downregulation of Sp proteins and / or ErbB2. Proteasome dependent degradation of Sp1, Sp3, Sp4 and ErbB2 will be determined using lactacystin to inhibit proteasomes. ErbB2 and their downstream proteins will be determined by western blot analysis. Sp protein mRNA levels will be determined by real-time PCR. Since a putative Sp binding site is found localized in the promoter region of YY1, an ErbB2 transcriptional activator, regulation of expression of YY1 and ErbB2 by Sp proteins will be determined by transfecting cells with siRNAs for Sp1, Sp3, and Sp4 or combination of the three Sp proteins, and the level of YY1 and ErbB2 proteins
will be determined by western blot analysis. A recent study showed that miR 27a targets ZBTB10, a repressor of Sp transcription factors in breast cancer cells [478]. Therefore, the effects of BA on miR27a and ZBTB10 expression will be determined by real-time PCR. Cannabinoid receptors have been reported as anticancer drugs targets and we hypothesized that BA may target Sp proteins and ErbB2 by activating cannabinoid receptors, and this will be determined by cotreatment of BA with cannabinoid receptor 1 and 2 (CB1 and CB2) antagonists, AM251 and AM630 respectively, and effects of BA on Sps, ErbB2, miR27a, and ZBTB10 will be determined using techniques described as above. Effects of BA in vivo will be determined using athymic nude mouse xenograft models.

**1.4.3 Objective 3: Mechanisms of aspirin action in breast cancer cells**

Aspirin usage lowers the incidence of multiple cancers and also inhibits tumor recurrence of colon and breast cancer patients who use aspirin after cancer diagnosis [416,417]. In this research, it was observed that aspirin also inhibits growth of ErbB2-overexpressing BT474 and MDA-MB-453 breast cancer cells and both Sp1, Sp3, Sp4 and ErbB2 proteins are downregulated in aspirin treated breast cancer cells. Therefore, the objective of this research will be to investigate the mechanisms of anti-cancer activity of aspirin in BT474 and MDA-MB-453 breast cancer cells and the contributions of aspirin-induced downregulation of Sp proteins and / or ErbB2 in mediating this response. Aspirin induced apoptosis in cells will be determined using terminal deoxynucleotidyl
transferase d-UTP nick end labeling (TUNEL) assay. Proteasome-dependent degradation of Sp1, Sp3, Sp4 and ErbB2 will be investigated using lactacystin to inhibit proteasomes; Sp proteins / ErbB2 and their downstream proteins will be detected using western blots. Phosphatase inhibitor cocktail II (PIC II) and sodium orthovanadate (Na$_3$VO$_4$) will be used to determine whether aspirin-induced downregulation of Sp1, Sp3, Sp4 and ErbB2 are dependent on induction of phosphatases. mRNA levels of specific phosphatases in aspirin treated cells will be determined using real-time PCR. Knocking down the specific phosphatase by RNAi will be conducted to determine the role of induced phosphatases in mediating downregulation of Sp1, Sp3, Sp4 and ErbB2.
2. THE NSAID TOLFENAMIC ACID INHIBITS BT474 AND SKBR3 BREAST CANCER CELL AND TUMOR GROWTH BY REPRESSING ErbB2 EXPRESSION*

2.1 Introduction

Breast cancer is one of the major causes of premature death in women; however, the combination of early detection coupled with improved treatment has significantly improved survival from this disease [479-481]. Antiestrogens and aromatase inhibitors are highly effective endocrine therapies used for treating early stage estrogen receptor (ER)-positive breast cancer. Compounds that include tamoxifen, raloxifene, fulvestrant, and their combinations or sequential use provide successful outcomes for patients with hormone-responsive tumors [480-485]. Later stage or less differentiated ER-negative breast cancers are more aggressive; patient survival is relatively low; and various therapeutic regimes are less effective [486-489]. Improvements in the effectiveness of chemotherapies have been obtained using drug combinations and differences in the timing of drug delivery [489]. In addition, newer mechanism-based anticancer drugs that target critical kinase, survival, growth promoting and angiogenic pathways are also promising new chemotherapies for treating breast and other tumor types [488,489].

Epidermal growth factor receptors (EGFRs) are receptor tyrosine kinases overexpressed in many cancers and ErbB2/HER2/neu is an oncogene overexpressed in 20-30% of all breast cancers. ErbB2-positive tumors tend to be aggressive with a poor prognosis for patient survival, and the recombinant monoclonal antibody trastuzumab (Herceptin) has been used as a single agent and in combination therapy for successfully treating patients with breast tumors overexpressing ErbB2 [490-493]. Since Herceptin targets the extracellular domain of ErbB2, there is a decrease in receptor tyrosine kinase activity and various downstream targets that are important for ErbB2-dependent tumor growth and survival. For example, treatment of breast cancer cells overexpressing ErbB2 with Herceptin decreased ErbB2 phosphorylation and also mitogen-activated protein kinase (MAPK)- and phosphatidylinositol-3-kinase (PI3K)-dependent phosphorylation of MAPK and Akt, respectively [494].

Tolfenamic acid (TA) is a non-steroidal anti-inflammatory drug (NSAID) used for treatment of migraine headaches and alcohol-induced hangovers [495]; however, recent studies have demonstrated the efficacy of this drug for cancer chemotherapy [413,496]. TA inhibits pancreatic cancer cell growth in vitro and tumor growth in vivo through inducing proteasome-dependent degradation of Sp1, Sp3 and Sp4 proteins which are overexpressed in these cells and tumors [369,413,496]. The effectiveness of TA is associated with repression of Sp proteins and Sp-dependent genes such as vascular endothelial growth factor (VEGF) and VEGF receptor 1 (VEGFR1). The antiangiogenic activity of TA
correlated with the inhibition of liver metastasis in an orthotopic model for pancreatic cancer [495]. In this study, we show that TA also inhibits growth of ErbB2-overexpressing BT474 and SKBR3 breast cancer cells; however, this is not accompanied by a coordinate repression of Sp proteins. Inhibition of ErbB2-overexpressing breast cancer cell and tumor growth by TA is associated with downregulation of ErbB2. This novel observation highlights the possibility that ErbB2-overexpressing breast tumors and tumors derived from other tissues may be targeted by TA and structurally-related NSAIDs that exhibit relatively low toxicity.

2.2 Materials and methods

Chemicals, antibodies, plasmids, and reagents - Tolfenamic acid, mefenamic acid, flufenamic acid, N-flumic acid and diclofenac were purchased from LKT Laboratories, Inc. (St. Paul, MN). Lactacystin, cycloheximide and β-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against ErbB2 (C-18), Sp1 (PEP2), Sp3 (D-20), Sp4 (V-20), Akt (H-136), p-Akt (Ser473), MAPK(C-14), p-MAPK (E-4), cyclin D1 (M-20), p27 (C-19), PEA3 (16), AP-2α (C-18 and 3B5) and YY1 (H-10) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); antibody against ErbB2 (Ab-3) was obtained from Calbiochem (San Diego, CA); and antibody against EEA1 was obtained from Upstate (Lake Placid, NY). The pErbB2-500 construct was kindly provided by Dr. Christopher C. Benz (University of California, San Francisco, CA) and the full length AP-2 cDNA construct TFAP2A was purchased from Open Biosystem
Reported lysis buffer and luciferase reagent for luciferase studies were purchased from Promega (Madison, WI). β-Galactosidase (β-gal) reagent was obtained from Tropix (Bedford, MA). LipofectAMINE reagent was supplied by Invitrogen (Carlsbad, CA). Western lightning chemiluminescence reagent was from Perkin-Elmer Life Sciences (Boston, MA).

Cell lines - Human mammary carcinoma cell lines MDA-MB-231, MCF-7, BT474 and SKBR3 were obtained from the American Type Culture Collection (Manassas, VA). Cell lines were cultured with 10% fetal bovine serum (FBS) in DMEM (BT474, MDA-MB-231 and MCF-7) or McCoy’s 5A medium (SKBR3). Cells were maintained at 37°C in the presence of 5% CO₂.

Cell proliferation assay - Cells (2-3 x 10⁴ per well) were plated in 12-well plates and allowed to attach for 24 hr. The medium was then changed to DMEM/Ham’s F-12 medium containing 2.5% charcoal-stripped FBS, and either vehicle (DMSO) or different concentrations of TA were added. Fresh medium and compounds were added every 48 hr, and cells were then trypsinized and counted at the indicated time points using a Coulter Z1 cell counter. Each experiment was done in triplicate, and results are expressed as means ± SE for each set of experiments.

Western blotting - Cells were rinsed with PBS and collected by scraping cells from the culture plate in 200 µL of lysis buffer. The cell lysates were incubated on ice for 1 hr with intermittent vortex mixing and then centrifuged at 40,000 g for 10 min at 4°C. Equal amounts of protein were separated on SDS-
polyacrylamide gels. Proteins were transferred to Immobilon P membranes (Millipore, Bedford, MA) using a Bio-Rad Trans-blot apparatus and transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS, 20% methanol). After blocking in TBST-Blotto [10 mmol/L Tris-HCl, 150 mmol/L NaCl (pH 8), 0.05% Triton X-100, 5% nonfat dry milk] for 30 min, the membranes were incubated with primary antibodies overnight at 4°C and then with horseradish peroxidase-conjugated secondary antibody for 2 hr at room temperature. Proteins were visualized using the chemiluminescence substrate (Perkin-Elmer Life Sciences) for 1 min and exposed to Kodak X-OMAT AR autoradiography film (Eastman Kodak, Rochester, NY). For protein quantitation, band intensities were normalized to β-actin (loading control) and compared to band intensities for the DMSO (control) set at 1.0 or 100%.

Quantitative real-time PCR - Total RNA was purified using RNeasy Mini Kit (Qiagen, Germantown, MD) and cDNA was prepared using Reverse Transcription System (Promega, Madison, WI). Each PCR was carried out in triplicate in a 30 µL volume using SYBR Green Mastermix (Applied Biosystems, Foster City, CA) for 15 min at 95°C for initial denaturing, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min in the Applied Biosystems 7900HT Fast Real-time PCR System. The ABI Dissociation Curves software was used following a brief thermal protocol (95°C for 15 s and 60°C for 15 s, followed by a slow ramp to 95°C) to control for multiple species in each PCR amplification. Values for each gene were normalized to expression levels of TATA-binding protein (TBP).
Primers were obtained from Integrated DNA Technologies. The following primers were used:

- **hNeu(F):** 5'-ACC GGC ACA GAC ATG AAG CT-3'.
- **hNeu(R):** 5'-AGG AAG GAC AGG CTG GCA TT-3'.
- **TBP (F):** 5'-TGC ACA GGA GCC AAG AGT GAA-3'.
- **TBP (R):** 5'-CAC ATC ACA GCT CCC CAC CA-3'.

**DNA transfection and luciferase assays** - Cells were plated in 12-well plates at 1 x 10^5 per well and cultured as described above. After growth for 16 to 20 hr, transfections were done by using LipofectAMINE (Invitrogen) according to the manufacturer's protocol. After 5 hr of transfection, the transfection mix was replaced with complete media containing either vehicle (DMSO) or TA for 20 to 22 hr. Cells were then lysed with 100 μL of 1x reporter lysis buffer, and 30 μL of cell extract were used for luciferase and β-gal assays. LumiCount was used to quantitate luciferase and β-gal activities, and the luciferase activities were normalized to β-gal activity.

**Immunofluorescence microscopy** - Cells were fixed immediately in 4% paraformaldehyde, added with 0.3% Triton X-100 (Roche Molecular Biochemicals, Indianapolis, IN) for 10 min, permeabilized in PBS with 0.3% Triton X-100 for 10 min, and preincubated for 1 hr with 10% normal goat serum (Vector Laboratories, Burlingame, CA). Cells were incubated with anti-ErbB2 antibody (1:80) or anti-EEA1 antibody (1:200) overnight and incubated with FITC-conjugated or Cy5-conjugated secondary antibody (1:200; Chemicon,
Temecula, CA) for 1 hr. The two-well chambers were mounted with mounting medium (Vector Laboratories). The slides were viewed using an LSM 510 Meta confocal microscope (Carl Zeiss, Jena, Germany) equipped with 40X and 63X objectives. Images were analyzed and processed using the LSM software v. 3.2 (Carl Zeiss) and occasionally Adobe Photoshop 7.0.

**Animals and orthotopic implantation of breast tumor cells** - Female ovariectomized athymic nu/nu mice (5-7 wk old) were purchased from the Animal Production Area of the National Cancer Institute Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed and maintained under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the NIH. Under anesthetic condition, a 0.72-mg 60-day release 17β-estradiol pellet (Innovative Research, Sarasota, FL) was implanted into the interscapular region of each mouse. One day later, BT474 cells (3×10⁶ cells) were injected s.c. under the mammary fat pad area of each mouse. The tumor sites were monitored twice a week and when palpable (12 days), mice were randomized into two groups of 5 mice per group and dosed by oral gavage with corn oil or 25 mg/kg/d tolfenamic acid for 27 days. The mice were weighed, and tumor size was measured at the indicated time with calipers to permit calculation of tumor volumes: \( V = \frac{LW^2}{2} \), where \( L \) and \( W \) were length and width,
respectively. Final body and tumor weights were determined at the end of the dosing regimen, and tumor blocks were obtained for histopathologic analysis.

*Immunohistochemistry* - Tissue sections (4-5 μM thick) mounted on poly-L-lysine-coated slide were deparaffinized by standard methods. Endogenous peroxidase was blocked by the use of 2% hydrogen peroxide in PBS for 1 min. Antigen retrieval for ErbB2 and p-MAPK staining was done for 10 min in 10 mmol/L sodium citrate buffer (pH 6) heated at 95°C in a steamer followed by cooling at room temperature for 15 min. The slides were washed with PBS and incubated for 30 min at room temperature with a protein blocking solution (VECTASTAIN Elite ABC kit, Vector Laboratories, Burlingame, CA). Excess blocking solution was drained, and the samples were incubated overnight at 4°C with one of the following: a 1:60 dilution of ErbB2 antibody or a 1:80 dilution of p-MAPK, AP-2 and YY1 antibodies. Sections were then incubated with biotinylated secondary antibody followed by streptavidin (VECTASTAIN Elite ABC kit). The color was developed by exposing the peroxidase to diaminobenzidine reagent (Vector Laboratories), which forms a brown reaction product. The sections were then counterstained with Gill's hematoxylin. ErbB2, AP-2, YY-1 and p-MAPK expression were identified by the brown cytoplasmic staining. H&E staining was determined as previously described [369,413,496].

2.3 Results

*TA inhibits proliferation of BT474 and SKBR3 cells* - Figures 16A and 16B illustrate the effects of TA on proliferation of ErbB2-overexpressing BT474 and
SKBR3 breast cancer cell lines. The lowest concentration of TA (25 μM) had minimal effects, whereas 50 and 100 μM TA inhibited growth of BT474 and SKBR3 cells and IC$_{50}$ values were 41.5 and 52.5 μM, respectively. These results are similar to those previously reported in pancreatic cancer cells [413] and in ongoing studies in other cancer cell lines. The effects of TA and other substituted biphenylamine-1-carboxylic acids on proliferation of BT474 and SKBR3 cell was also determined (Figs. 16C and 16D). A comparison of the growth inhibitory effects of 50 μM TA, and structurally-related mefanamic acid, flufenamic acid, N-flumic acid, and diclofenac indicated that mefanamic acid was the least active among these 5 structurally related analogs. Differences among the other 4 substituted biphenyl-1-carboxylic acid NSAIDs were not large; however, TA was the most active compound in BT474 cells as previously observed for these compounds and was used as the prototype for the remaining studies.

**TA downregulates ErbB2 and ErbB2-dependent responses** - The growth inhibitory activity of TA and related compounds in cancer cells has been correlated with downregulation of Sp1, Sp3 and Sp4 proteins [369,413,496]; however, results in Figures 17A and 17B show that up to 100 μM TA did not appreciably affect Sp3 or Sp4 expression in BT474 or SKBR3 cells, and 75-100 μM TA decreased Sp1 protein only after treatment of BT474 cells for 72 hr. Sinc-
Figure 16. Effects of TA and related compounds on cell proliferation. TA-mediated inhibition of BT474 (A) and SKBR3 (B) cell growth. Cells were treated with different concentrations of TA for up to 6 days and the number of cells in each treatment group were determined as described in the Materials and Methods. Significant (p < 0.05) inhibition of cell growth was observed for 50 and 100 μM TA. Inhibition of BT474 (C) and SKBR3 (D) cell proliferation by TA and related compounds. Cells were treated with 50 μM TA and related compounds as described above and significant (p < 0.05) growth inhibition is indicated (*). Results are expressed as means ± SE for at least 3 replicate determinations for each treatment group.
Figure 17. Effects of TA on Sp, ErbB2 and ErbB2-dependent proteins. Sp protein expression in BT474 (A) and SKBR3 (B) cells treated with TA. Cells were treated with different concentrations of TA for up to 72 hr and whole cell lysates were analyzed by western blots as described in the Materials and Methods. ErbB2 and ErbB2-dependent protein expression in BT474 (C) and SKBR3 (D) cells treated with TA. Cells were treated as described in (A)/(B) and whole cell lysates were analyzed by western blots as described in the Materials and Methods. Bar graphs compare protein expression (normalized to β-actin) relative to DMSO (set at 1.0) and results are means ± SE for 3 replicate determinations for each treatment group. Significant (p < 0.05) decreases in protein expression are indicated (*).
ErbB2 is a major driving force for the growth and survival of both cell lines, we also examined the effects of TA or ErbB2 protein expression (Figs. 17C and 17D). TA induced a time- and concentration-dependent decrease in ErbB2 protein in BT474 cells and this was accompanied by decreased phosphorylation of MAPK and Akt but not their corresponding proteins. Cyclin D1 and p27 are two proteins down- and upregulated by ErbB2-dependent kinases, respectively [494], and results in Figure 18A show that TA also decreased cyclin D1 and increased p27 expression in BT474 and SKBR3 cells. Previous reports indicate that geldanamycin and ansamycins decrease ErbB2 protein through destabilizing interactions with chaperones resulting in enhanced proteasome-dependent degradation of ErbB2 [270, 497, 498]. Initial studies with the proteasome inhibitor MG-132 gave conflicting results since MG132 alone decreased ErbB2 protein in BT474 and SKBR3 cells (data not shown). Studies with lactacystin, another proteasome inhibitor showed that TA-induced downregulation of ErbB2 protein was not inhibited after cotreatment with lactacystin (Fig. 18B). Treatment of BT474 and SKBR3 with 50 μM TA for 2, 6 and 12 hr resulted in a time-dependent decrease in ErbB2 mRNA levels (Fig. 18C) and in cells transfected with pErbB2, a construct containing the -0.5 kB region from the ErbB2 promoter, TA also decreased luciferase activity in both cell lines (Fig. 18D). The results demonstrate that TA acts, in part, by decreasing ErbB2 transcription in BT474 and SKBR3 cells.
Figure 18. TA decreases ErbB2-dependent genes and ErbB2 gene expression. Effects of TA on cyclin D1/p27 (A) and ErbB2 (B) protein levels. BT474 and SKBR3 cells were treated with TA alone or TA in combination with 2 μM lactacystin for 48 or 72 hr as indicated, and whole cell lysates were analyzed by western blots as described in the Materials and Methods. (C) TA decreases ErbB2 mRNA levels. BT474 and SKBR3 cells were treated with DMSO or 50 μM TA for different times and ErbB2 mRNA levels were determined by RT-PCR as described in the Materials and Methods. Results are expressed as means ± SE for three replicate determinations for each treatment group and significantly (p < 0.05) decreased mRNA levels are indicated. (D) TA decreases luciferase activity. Cells were transfected with pErbB2, treated with DMSO, or 50 μM TA, and luciferase activity (relative to β-gal) was determined as described in the Materials and Methods. Results are expressed as means ± SE for 3 replicate determinations for each treatment group and significant (p < 0.05) decreases in activity are indicated (*).
Mechanisms of ErbB2 downregulation by TA - The mechanisms of TA-dependent inhibition of ErbB2 transcription were investigated in BT474 and SKBR3 cells treated with TA alone or in combination with the protein synthesis inhibitor cycloheximide (Fig. 19A). Cycloheximide did not affect TA-dependent ErbB2 mRNA downregulation in either cell line, suggesting that TA does not induce an inhibitory protein that acts on erbB2 transcription. However, in studies on erbB2 mRNA stability carried out in the presence or absence of the transcriptional inhibitor actinomycin D, TA significantly decreased erbB2 mRNA stability in BT474 cells over the 8 hr duration of this experiment (Fig. 19B). In contrast, only minimal effects were observed in SKBR3 cells, demonstrating cell context-dependent effects of TA on erbB2 mRNA stability. YY1 and AP-2 cooperatively regulate ErbB2 expression in ErbB2 overexpressing cells [321], and Figure 19C summarizes the effects of TA on expression of these transcription factors in BT474 and SKBR3 cells. TA decreased YY1 and AP-2 protein levels in BT474 and SKBR3 cells and, in cells cotreated with TA plus the proteasome inhibitor lactacystin, levels of YY1 and AP-2 were similar to those observed after treatment with TA alone. These results indicate that downregulation of these transcription factors was proteasome-independent.
Figure 19. Effects of TA on ErbB2 expression and transcriptional regulatory proteins. Effects of cycloheximide (A) and actinomycin D (B) on ErbB2 mRNA levels and stability in cells treated with TA. BT474 and SKBR3 cells were treated with 50 μM TA alone or in combination with cycloheximide or actinomycin D and ErbB2 mRNA levels were determined at various time points as described in the Materials and Methods. Results are expressed as means ± SE for 3 replicate determinations for each treatment group and significant (p < 0.05) decreases are indicated (*). (C) Effects of TA on YY1/AP-2 protein levels. BT474 and SKBR3 cells were treated with TA for the indicated times and whole cell lysates were analyzed by western blot analysis as indicated in the Materials and Methods. (D) AP-2 activates the ErbB2 promoter. BT474 cells were transfected with empty vector (PGL3) or the pErbB2-luc construct, and one treatment group was cotransfected with AP-2 expression plasmid. Luciferase activity was determined as described in the Materials and Methods. Results are means ± SE for 3 separate determinations and significant (p < 0.05) induction of luciferase activity by AP-2 is indicated (*). Western blot analysis of lysates demonstrates that the AP-2 expression plasmid increases AP-2 protein.
BT474 cells were transfected with PGL3 empty vector or the pErbB2-luc construct (Fig. 19D). In cells cotransfected with an AP-2 expression plasmid, there was a significant induction of luciferase activity demonstrating that AP-2 expression activates the ErbB2 promoter and demonstrates the importance of AP-2 for ErbB2 expression. We also investigated the effects of TA on subcellular localization of ErbB2 since many drugs that decrease ErbB2 protein induce ErbB2 delocalization from the plasma membrane into the cytoplasm [499-501]. In solvent (DMSO)-treated SKBR3 cells, ErbB2 staining was primarily on the plasma membrane (Fig. 20), whereas the endosome marker, early endosome antigen 1 (EEA1), staining was on the endosome and did not colocalize with ErbB2. After treatment with 50 μM TA for 24 hr, the ErbB2 plasma membrane staining was observed and, in the merge of both ErbB2 and EEA1, it was evident that TA did not significantly induce internalization of erbB2 as reported for other agents [499-501]. Similar results were observed in BT474 cells demonstrating that TA did not induce internalization and subsequent degradation of ErbB2 in the ErbB2 overexpressing breast cancer cell lines.
Figure 20. Immunostaining of ErbB2. SKBR3 and BT474 cells were treated with 50 μM TA for 24 hr and cells were fixed, immunostained with ErbB2 or EEA1 antibodies and analyzed by confocal microscope and softwares as described in the Materials and Methods.
TA inhibits tumor growth in athymic nude mice bearing BT474 xenografts

- We also investigated the in vivo antitumorigenic activity of TA (20 mg/kg/d) which was administered orally by gavage to female athymic nude mice bearing BT474 cells injected into the mammary fat pad. Tumor size was determined over the treatment period (Fig. 21A) and there was a significant decrease in mice treated with TA compared to those treated with the solvent alone. In addition, TA also decreased mammary tumor weight compared to solvent treated animals (Fig. 21B). H & E staining (Fig. 21C) shows that tumors from untreated mice consisted of nests of cells in a semi-organized fashion with nuclear molding and high nuclear to cytoplasmic ratio. In addition, cells with marked atypical features such as anisocytosis, anisokaryosis, and multiple variably sized nucleoli were also noted. Tumors from tolfenamic acid-treated mice consisted of neoplastic cells similar to that noted from the untreated mice. However, the nests of tumor cells were highly disorganized with multiple nuclear fragmentations and condensations; in addition, epithelial atypia was decreased. Treatment of mice with TA also decreased expression of ErbB2, phospho-MAPK, AP-2 and YY-1 in tumors compared to levels in tumors from mice treated
Figure 21. TA inhibits tumor (BT474 xenografts) growth. Inhibition of tumor volume (A) and weight (B). Athymic nude mice bearing BT474 cells as xenografts were treated with TA (25 mg/kg/d) and tumor volumes and weights were determined as described in the Materials and Methods. Significantly (p < 0.05) decreased tumor weights are indicated (*). (C) H&E staining and immunostaining. Tumors from vehicle control (corn oil)- and TA-treated mice were fixed and stained (H&E) and immunostaining for ErbB2, phospho-MAPK, YY1 and AP-2 as described in the Materials and Methods. (D) A schematic model summarizing the effects of TA on ErbB2 in BT474 and SKBR3 cells.
with corn oil (Fig. 21C). Thus, results of both *in vivo* and *in vitro* data show that TA inhibits tumor and cancer cell growth through downregulating ErbB2 expression, suggesting that this relatively non-toxic NSAID may represent a novel clinical approach for treatment of cancers that overexpress this oncogene.

As a control for this experiment, we also observed that TA significantly decrease growth of MDA-MB-231 and MCF7 breast cancer cells (Fig. 22A), and mefanamic acid decreased ErbB2 and phosphorylation of MAPK and Akt but at higher concentrations than required for TA (Fig. 22B). Thus, TA specifically decreased expression of ErbB2 protein and ErbB2-dependent phosphorylation pathways in BT474 and SKBR3 cells. We also observed some treatment-related changes in MAPK and Akt proteins (Fig. 22C) in BT474 cells and this was particularly evident for MAPK (but not Akt) after treatment for 72 hr. Previous studies show that the transcription factor PEA3 suppresses ErbB2 expression [316,502]. Therefore, we investigated the effects of TA on PEA3 expression in BT474 and SKBR3 cells (Fig. 22D) and did not observe any changes in expression of this transcription factor.
Figure 22. Supplemental figure for chapter 2. A. The dose- and time-dependent effects of TA on proliferation of MDA-MB-231 and MCF-7 cells. B. The effects of 50 μM mefanamic acid on proliferation of BT474 cells and the effects of higher concentrations on ErbB2 protein and phosphorylation of Akt and MAPK. C. Quantitation of specific bands in Figure 17C. D. Effects of TA on PEA3 protein expression in BT474 and SKBR3 cells.
2.4 Discussion

The development of Herceptin as a biotherapy for ErbB2-overexpressing breast cancer patients has been an important innovation for treating this subset of individuals [490-493]. Moreover, combination therapy of Herceptin plus other drugs including paclitaxel are also being used as adjuvant therapy for breast cancer. Herceptin is not without side effects, and cardiotoxicity has been reported in a small number of patients [493]. Based on the success of targeting ErbB2 for cancer chemotherapy, other chemotherapeutic agents have been developed for blocking activity of this receptor, and these include both selective and non-selective tyrosine kinase inhibitors, geldanamycin and compounds that interfere with chaperones such as heat shock protein 90, fatty acid synthase (FAS) inhibitors, and orlistat, a drug used in weight loss [220,270,492-494,497-501,503-508]. These compounds all block activation of ErbB2 and ErbB2-dependent downstream responses, although their overall mechanisms of action are highly variable. TA is a relatively non-toxic NSAID used for treatment of migraine headaches in humans, and TA has multiple applications in veterinary medicine. Development of this drug for cancer chemotherapy is promising due to the relatively low toxicity of TA and related compounds. Previous studies in this laboratory reported that the anticancer activity of TA in pancreatic cancer cell lines is associated with their repression of Sp proteins and Sp-dependent genes [369,413,496], and we hypothesized that TA may be effective in treatment of ErbB2-positive breast cancer through a comparable mechanism.
Sp proteins are overexpressed in ER-positive and ER-negative breast cancer cell lines including SKBR3 and BT474 cells [478], and Figure 16 shows that TA inhibits growth of both cell lines with potencies similar to that observed for this compound in pancreatic cancer cells [369,413,496]. However, treatment of SKBR3 and BT474 cells with TA did not appreciably affect Sp1, Sp3 and Sp4 protein levels, although we did observe a consistent 20-30% decrease in Sp1 in BT474 cells treated with 75-100 µM TA for 3 days. These results contrast to ongoing studies in pancreatic and other cancer cell lines where TA decreases Sp1, Sp3 and Sp4 proteins (data not shown). However, further analysis of protein expression in BT474 or SKBR3 cells treated with TA showed that ErbB2 protein expression was decreased (Fig. 17) and these results are consistent with the growth inhibitory effects of TA in cells, where their growth and survival are ErbB2-dependent. Herceptin and other classes of drugs that block phospho-ErbB2 formation/activation or degrade ErbB2 exhibit similar effects on ErbB2-dependent downstream responses including decreased phosphorylation of Akt and MAPK, downregulation of cyclin D1 and induction of p27 [220,494,499-501,503-508]. Figures 17C, 17D and 18A illustrate that TA also exhibits an identical pattern of responses in BT474 and SKBR3 cells which is consistent with TA-dependent downregulation of ErbB2 protein.

We also compared the effects of TA with other agents that block ErbB2 signaling. Tyrosine kinase inhibitors such as ZD1839 may or may not affect ErbB2 expression but, in the short term, their effects are primarily on decreased
ErbB2 phosphorylation [220,494,503-505]. In contrast, ansamycins, proteasome inhibitors such as bortezomib, and FAS synthase inhibitors all decrease ErbB2 protein expression in BT474 and/or SKBR3 cells [270,497-501,506-508], and similar responses were observed for TA (Figs. 17C and 17D). However, in contrast to FAS inhibitors [508], TA did not induce PEA3 which inhibits ErbB2 expression at the transcriptional level (Fig. 19C). Ansamycins such as geldanamycin induce proteasome dependent degradation of ErbB2 [270,497-499], whereas TA-induced repression of ErbB2 protein was proteasome-independent (Fig. 18A). Interestingly, geldanamycin, the proteasome inhibitor bortezomib (Valcade), and the reversible tyrosine kinase inhibitor CI-1033 decrease ErbB2 expression and this is associated, in part, with intracellular localization of ErbB2 from the cell membrane and this process is related to the subsequent decrease in ErbB2 protein [499,500]. In contrast, treatment with TA did not induce translocation of cell membrane ErbB2 into the cell (Fig. 20), indicating that the mechanism of TA-dependent downregulation of ErbB2 is different from these classes of drugs.

TA clearly affected ErbB2 transcription and decreased ErbB2 mRNA levels (Fig. 18C) and promoter activity in BT474 and SKBR3 cells transfected with the pErbB2 constructs that contained a -0.5 kB promoter insert (Fig. 18D). The protein synthesis inhibitor cycloheximide did not affect TA-induced repression of ErbB2 mRNA levels (Fig. 19A), suggesting that an induced "inhibitory" protein was not involved. These results were consistent with the
failure of TA to induce PEA3 (Fig. 22D) which inhibits ErbB2 expression [316,502] and plays a role in the reported downregulation of ErbB2 gene expression by inhibitors of FAS cells [506-508]. A previous study showed that YY1 and AP-2 transcription factors cooperatively regulate ErbB2 expression in BT474 and other cancer cell lines [321]. Figure 19C illustrates that TA decreased expression of YY1 and AP-2 transcription factors in both BT474 and SKBR 3 cells after treatment for 48 hr, and this response was not reversed by the proteasome inhibitor lactacystin. The role of AP-2 in basal expression of ErbB2 in BT474 cells is illustrated in Figure 19D showing that overexpression of AP-2 activates the ErbB2 promoter. Thus, TA-induced downregulation of ErbB2 protein and mRNA levels was due to the effects of this compound on AP-2 and YY1 expression in both cell lines, and decreased ErbB2 mRNA stability (Fig. 19B) also contributed to these effects in BT474 cells. It is also possible that TA affects expression of other factors in BT474 and SKBR3 cells that decrease ErbB2 transcription and these are currently being investigated.

The effects of TA on SKBR3 and BT474 cell growth and on ErbB2 in vitro were also observed in athymic nude mice injected with BT474 cells into mammary fat pads (Fig. 21). TA decreased tumor growth and weight and downregulated ErbB2 protein and ErbB2-dependent responses (phospho-MAPK). TA also decreased immunostaining of both AP-2 and YY-1 in TA-treated tumors (compared to corn oil-treated) and these in vivo results complemented the cell culture studies. Thus, the anticarcinogenic activity of TA
is associated with downregulation of Sp transcription factors in some cell lines [369,413,496] and repression of the oncogene ErbB2 in breast cancer cell lines overexpressing this oncogene. The mechanisms of action of TA are, in part, cell context-dependent since this NSAID decreases ErbB2 mRNA stability in BT474 and not SKBR3 cells and this may involve differential effects on factors that control mRNA stability (Fig. 21D). However, the critical TA-dependent effects in both cell lines involves downregulation of YY1 and AP-2 (which regulate ErbB2 expression), whereas proteasome-dependent degradation of ErbB2, induction of a repressor such as PEA3 or enhanced ErbB2 endocytosis are not involved in downregulation of ErbB2 by TA (Fig. 21D). The mechanisms of TA-induced repression of YY-1 and AP-2 and the potential clinical applications for TA in treatment of ErbB2 overexpressing cancers are currently being investigated.
3. BETULINIC ACID INHIBITS ErbB2-OVEREXPRESSING BREAST CANCER CELL AND TUMOR GROWTH BY ACTIVATION OF CANNABINOID RECEPTOR-DEPENDENT REPRESSION OF Sp TRANSCRIPTION FACTORS

3.1 Introduction

Naturally-occurring triterpenoids and triterpenoid-derived compounds have been widely used in traditional medicines and for treatment of various diseases [509-511]. Activities of these compounds are structure-dependent, however many triterpenoids and their synthetic derivatives are potent anti-inflammatory agents and exhibit anticancer activities against several tumor types. For example, 2-cyano-1,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and related compounds are synthesized from oleanolic acid, a triterpenoid found in various plants and herbs, and are potent anti-inflammatory drugs and anticancer agents [510,512]. Betulinic acid (BA) is a component of bark extracts and is readily synthesized from betulin which can constitute up to 30% by weight of birch bark [444,513]. BA and several derivatives exhibit a broad spectrum of pharmacological properties including antiviral, antibacterial, anti-inflammatory, antimalarial and anticancer activities [444,513].

BA is relatively non-toxic in rodent studies and highly effective against melanoma in both in vivo and in vitro assays [460]. Subsequent research in several laboratories indicates that BA inhibits growth of multiple tumor types.
including breast cancer [444,513]. Studies in this laboratory show that BA inhibits prostate cancer cell and tumor growth in a xenograft model and one of the underlying mechanisms of action is due to BA-induced proteasome-dependent degradation of specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4 [448]. These proteins are highly expressed in several different cancer cell lines and tumors [413,448,475,478,514,515] whereas Sp1 levels in non-tumor tissue of rodents and humans is relatively low and decreases with age [516,517]. Results of RNA interference studies have identified several Sp-regulated genes and these include vascular endothelial growth factor (VEGF) and its receptors (VEGFR1 and VEGR2), epidermal growth factor receptor (EGFR, HER1), p65 unit of NFkB, survivin and hepatocyte growth factor receptor (c-MET) [369,475-477,496,515,518] and these genes are themselves individual targets for new mechanism-based drugs. Ongoing studies have identified several other anticancer agents that downregulate Sp transcription factors in cancer cells and these include the non-steroidal anti-inflammatory drug (NSAID) tolfenamic acid, arsenic trioxide, curcumin, the methyl ester of CDDO (CDDO-Me) [519] and a structurally-related compound synthesized from glycyrrhcinic acid, methyl 2-cyano-3,11-dioxo-18β-olean-1,12-dien-30-oate (CDODA-Me) [369,407,413,475-478,496,514,515,518-520]. The mechanisms of action of these anticancer drugs involve both proteasome-dependent and independent pathways which depend on the specific agent and cell context.
In this study we have used BA as a model to investigate the effect of Sp1, Sp3 and Sp4 downregulation on BT474 and MDA-MD-453 breast cancer cells that express the oncogene EGFR2 (ErbB2, HER2). BA inhibited cell and tumor growth in a xenograft model (BT474 cells) and BA also decreased expression of Sp1, Sp3, Sp4, ErbB2 and ErbB2-regulated kinases. Unlike EGFR, ErbB2 is not directly regulated by Sp transcription factors however BA-mediated repression of ErbB2 was due to downregulation of YY-1, an Sp-regulated gene that activates ErbB2 expression. In addition, we show for the first time that BA-mediated downregulation of Sp transcription factors and ErbB2 is dependent on activation of the cannabinoid receptor (upstream), downregulation of miR-27a and induction of ZBTB10, an Sp repressor which has previously been identified as an inhibitor of Sp1, Sp3, Sp4 and Sp regulated gene expression [478,514,519,521].

3.2 Materials and methods

**Chemicals, antibodies, plasmids, and reagents** - Betulinic acid, lactacystin were purchased from Sigma-Aldrich (St. Louis, MO). AM 251, AM 630, capsazepine and WIN-55,212-2 were purchased from Tocris Bioscience (Ellisville, MO). Antibodies against ErbB2 (C-18), p-ErbB2 (Try 1248)-R, Sp1 (PEP2), Sp3 (D-20), Sp4 (V-20), Akt (H-136), p-Akt (Ser473), MAPK(C-14), p-MAPK (E-4), β-actin (C4), AP-2α (C-18 and 3B5), YY1 (H-10), CB1 (H-150) and CB2 (H-60) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against cleaved-PARP (D214) and survivin were purchased from Cell
Signaling Technology (Danvers, MA). The YY1 promoter plasmids (YY-1 p-1729 Luc and YY-1 p-277 Luc) was kindly provided by Dr. Ed Seto (University of South Florida, Tampa, FL). The ZBTB10 expression vector and empty vector (pCMV6-XL4) were purchased from Origene (Rockville, MD). The wild-type (wt) or mutated (mt) miR-27a target sequence from the 3’-UTR of ZBTB10 was cloned into NotI and XhoI sites of psiCHECK2 dual luciferase reporter construct (Promega, Madison, WI) as described [522]. Reporter lysis buffer, luciferase reagent and Dual-Luciferase Reporter Assay Kit for luciferase studies were purchased from Promega (Madison, WI). β-Galactosidase (β-gal) reagent was obtained from Tropix (Bedford, MA). LipofectAMINE 2000 was supplied by Invitrogen (Carlsbad, CA). Western lightning chemiluminescence reagent was from Perkin-Elmer Life Sciences (Boston, MA).

**Cell lines** - Human mammary carcinoma cell lines BT474 and MDA-MB-453 were obtained from the American Type Culture Collection (Manassas, VA). Cell lines were cultured with 10% fetal bovine serum (FBS) in DMEM and were maintained at 37°C in the presence of 5% CO2.

**Cell proliferation assay** - Cells (2-3 x 10^4 per well) were plated in 12-well plates and allowed to attach for 24 hr. Then cells were treated with either vehicle (DMSO) or different concentrations of betulinic acid for up to 4 days. Fresh medium and compounds were added every 48 hr, and cells were then trypsinized and counted at the indicated time points using a Coulter Z1 cell
counter. Each experiment was done in triplicate, and results are expressed as means ± SE for each set of experiments.

**Western blotting** - Cells were rinsed with PBS and collected by scraping cells from the culture plate in 200 μL of lysis buffer. The cell lysates were incubated on ice for 1 hour with intermittent vortex mixing and then centrifuged at 40,000 g for 10 min at 4°C. Equal amounts of protein were separated on SDS-polyacrylamide gels. Proteins were transferred to Immobilon P membranes (Millipore, Bedford, MA) using a Bio-Rad Trans-blot apparatus and transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS, 20% methanol). After blocking in TBST-Blotto [10 mmol/L Tris-HCl, 150 mmol/L NaCl (pH 8), 0.05% Triton X-100, 5% nonfat dry milk] for 30 min, the membranes were incubated with primary antibodies overnight at 4°C and then with horseradish peroxidase-conjugated secondary antibody for 2 hr at room temperature. Proteins were visualized using the chemiluminescence substrate (Perkin-Elmer Life Sciences) for 1 min and exposed to Kodak X-OMAT AR autoradiography film (Eastman Kodak, Rochester, NY). For protein quantitation, band intensities were normalized to β-actin (loading control) and compared to band intensities for the DMSO (control) set at 1.0 or 100%.

**TUNEL assay** - Cells were plated in Lab-Tek II Chamber Slide System (Nalge Nunc International, Naperville, IL) and allowed to attach for 24 hr. Cells were then treated with either vehicle (DMSO) or different concentrations of BA for 24 hr, TUNEL staining was performed using the DeadEnd™ Fluorometric
TUNEL System (Promega, Madison, WI) following manufacture’s protocol. The slides were analyzed using a Nikon ECLIPSE 80i confocal microscope (Japan).

**Real-time PCR analysis of mRNAs and miRNAs** - Total mRNA was isolated using the RNeasy Protect Mini kit (Qiagen) according to the manufacturer’s protocol. RNA was reverse transcribed using Transcription System (Promega, Madison, WI) according to the manufacturer’s protocol. Each PCR was carried out in triplicate in a 30 μL volume using SYBR Green Mastermix (Applied Biosystems, Foster City, CA) for 15 min at 95°C for initial denaturing, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min in the Applied Biosystems 7900HT Fast Real-time PCR System. The ABI Dissociation Curves software was used following a brief thermal protocol (95°C for 15 s and 60°C for 15 s, followed by a slow ramp to 95°C) to control for multiple species in each PCR amplification. Values for each gene were normalized to expression levels of TATA-binding protein (TBP). Total miRNA was extracted using the mirvana miRNA isolation kit (Ambion). Quantification of miRNA (RNU6B, miRNA-27a) was done using the Taqman miRNA kit (Applied Biosystems) according to the manufacturer’s protocol with real-time PCR. U6 small nuclear RNA was used as a control to determine relative miRNA expression. Primers for Sp3 and Sp4 were purchased from Qiagen. Other primers were obtained from Integrated DNA Technologies:

\[
\text{Sp1 (F): } 5’-\text{TCA CCT GCG GGC ACA CTT-3’}.
\]
\[
\text{Sp1 (R): } 5’- \text{CCG AAC GTG TGA AGC GTT -3’}. 
\]
DNA and siRNA transfection - Cells were plated in 12-well plates at 1 x 10^5 per well and cultured as described above. After growth for 16 to 20 hr, transfections were carried out using Lipofectamin 2000 (Invitrogen) according to the manufacturer’s protocol. Transfection reagent was not removed from the cells and siRNA transfected cells were incubated for 72 hr; DNA or antisense RNA transfected cells were incubated for 48 hr at 37°C before experimentation. The negative control siRNA was purchased from Ambion (Austin, TX). siRNAs for Sp1 (Sp1-1: SASI_Hs02_00363664; Sp1-2: SASI_Hs01_00070994), Sp3 (5’-GCG GCA GGU GGA GCC UUC ACU TT-3’), Sp4 (5’-GCA GUG ACA CAU UAG UGA GCT T-3’), and YY1 (YY1-1: SASI_Hs01_00155071, YY1-2: SASI_Hs01_--155072) were purchased from Sigma-Aldrich (St. Louis, MO). iLamin (5’-CUG GAC UUC CAG AAG AAC ATT-3’), miR-27a mimic and as-miR-27a were purchased from Dharmacon RNA Technologies (Lafayette, CO). 100nM siRNAs were used in this study.

Luciferase assay - Transfected cells were lysed with 100 μL of 1x reporter lysis buffer, and 30 μL of cell extract were used for luciferase and β-gal assays. Lumicount was used to quantitate luciferase and β-gal activities, and the luciferase activities were normalized to β-gal activity.
**Animals and xenograft study** - Female ovariectomized athymic nu/nu mice (5-7 wk old) were purchased from the Harlan Laboratories (Indianapolis, IN). The mice were housed and maintained under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the NIH. Under anesthetic condition, BT474 cells ($1 \times 10^6$) were implanted with matrigel (BD Biosciences, San Jose, CA) s.c. into the flank of each mouse. Ten days later, mice were randomized into two groups of 6 mice per group and dosed by oral gavage with corn oil or 20 mg/kg/d of betulinic acid every other day for 28 days (14 doses). The mice were weighed, and tumor size was measured at the indicated time with calipers. Final body and tumor weights were determined at the end of the dosing regimen, and tumor blocks were obtained for histopathologic analysis.

**Immunohistochemistry** - Tissue sections (4-5 µM thick) mounted on poly-L-lysine-coated slide were deparaffinized by standard methods. Endogenous peroxidase was blocked by the use of 2% hydrogen peroxide in PBS for 1 min. Antigen retrieval for ErbB2 and Sp1 staining was done for 10 min in 10 mmol/L sodium citrate buffer (pH 6) heated at 95°C in a steamer followed by cooling at room temperature for 15 min. The slides were washed with PBS and incubated for 30 min at room temperature with a protein blocking solution (VECTASTAIN Elite ABC kit, Vector Laboratories, Burlingame, CA). Excess blocking solution
was drained, and the samples were incubated overnight at 4°C with one of the following: a 1:60 dilution of ErbB2 antibody or a 1:100 dilution of Sp1 antibody. Sections were then incubated with biotinylated secondary antibody followed by streptavidin (VECTASTAIN Elite ABC kit). The color was developed by exposing the peroxidase to diaminobenzidine reagent (Vector Laboratories), which forms a brown reaction product. The sections were then counterstained with Gill's hematoxylin. ErbB2 and Sp1 expression were identified by the brown staining.

*Competitive receptor binding* - Membrane proteins (100 or 25 μg) prepared from mouse brain or CHO-hCB2 cells were used in each binding assay, respectively. Increasing concentrations of the non-radioactive test ligands were incubated with 0.2 nM of the non-selective CB1/CB2 agonist [3H] CP-55,940 for 90 min at room temperature, and reactions were terminated by rapid vacuum filtration through Whatman GF/B glass fiber filters followed by three washes with ice-cold binding buffer as described previously [523]. Analysis of the binding data was performed using the non-linear regression (Curve Fit) function of GraphPad Prism® v4.0b to determine the concentration of the drug that displaced 50% of [3H]CP-55,940 (IC50), and measure of affinity (Ki) was derived from the IC50 values utilizing the Cheng-Pruschoff equation [524].

**3.3 Results**

*BA inhibits growth, induces apoptosis and downregulates Sp1, Sp3 and Sp4 in BT474 and MDA-MB-453 cells* - BT474 and MDA-MB-453 cells overexpress ErbB2 and these cell lines are widely used as models for
understanding the molecular mechanisms associated with drugs that target cancer cells expressing high levels of ErbB2. BA inhibited proliferation of both BT474 and MDA-MB-453 cells and the overall decrease in cell number was both concentration-(1, 5 and 10 µM) and time-(2 or 4 days) dependent and MDA-MB-453 cells were less responsive to BA than BT474 cells (Figs. 23A and 23B). The growth inhibitory effects of BA were also accompanied by induction of cleaved PARP, a marker of apoptosis and decreased expression of survivin an inhibitor of apoptosis was also observed (Fig. 23C). Induction of apoptosis by BA in BT474 and MDA-MB-453 was also confirmed in a TUNEL assay in which BA increased TUNEL staining in both cell lines (Fig. 23D).

The growth inhibitory effects of BA in LNCaP prostate cancer cells have been linked, in part, to activation of proteasome-dependent degradation of Sp1, Sp3 and Sp4 proteins [448] and results illustrated in Figures 24A and 24B show that after treatment of BT474 and MDA-MB-453 cells with 10 µM BA for 48 hr. there was a decrease in expression of Sp1, Sp3, Sp4 and survivin (an Sp-regulated gene) proteins in both cell lines. The proteasome inhibitor MG132 alone was cytotoxic to BT474 and MDA-MB-453 cells and MG132 alone decreased Sp proteins whereas lactacystin was not toxic and did not affect Sp protein expression (data not shown). Treatment of BT474 or MDA-MB-453 cells with 10 µM BA plus 1 µM lactacystin for 48 hr. resulted in decreased expression
Figure 23. Effects of BA on cell proliferation and apoptosis. BA-mediated inhibition of BT474 (A) and MDA-MB-453 (B) cell growth. Cells were treated with different concentrations of BA for up to 4 days and the number of cells in each treatment group was determined as described in the Materials and Methods. Significant (p < 0.05) growth inhibition is indicated (*). Results are expressed as means ± SE for at least 3 replicate determinations for each treatment group. C. Effects of BA on cleaved-PARP and survivin. Cells were treated with 10 μM BA for 48 hr and whole cell lysates were analyzed by western blots as described in the Materials and Methods. D. BA induces apoptosis in cancer cells. Cells were treated with DMSO or 10 μM BA for 24 hr and analyzed with a TUNEL assay as described in the Materials and Methods.
of Sp1, Sp3 Sp4 and survivin proteins that was not blocked after cotreatment with lactacystin suggesting that BA-mediated downregulation of Sp proteins was proteasome-independent. Therefore the effects of 10 µM BA on Sp1, Sp3 and Sp4 mRNA levels were investigated and after treatment of BT474 and MDA-MB-453 for 16 hr there was a significant decrease in Sp1, Sp3 and Sp4 mRNA levels (Fig. 24B), suggesting that the mechanism of action of BA involved transcriptional repression.

**BA downregulates ErbB2 and ErbB2-regulated genes** - Since ErbB2 plays a major role in the proliferation of BT474 and MDA-MB-453 cells the effects of BA alone and in combination with lactacystin on ErbB2, MAPK, Akt and their phosphorylated analogs were investigated. BA alone decreased ErbB2, p-ErbB2, and downstream kinases MAPK, p-MAPK, Akt and p-Akt expression (Fig. 24C) and these effects were not reversed after co-incubation with the proteasome inhibitor lactacystin. We also investigated the effects of BA alone and BA-plus lactacystin on expression of YY1 which is a key upstream regulator of ErbB2 in breast cancer cells overexpressing this oncogene [321]. The results showed that BA decreased expression of YY1 in both cell lines in the presence or absence of lactacystin (Fig. 24C). The linkage between Sp transcription factors and ErbB2 could be due to regulation of YY1 by Sp transcription since the YY1 promoter contains multiple GC-rich Sp binding sites [525]. We also investigated the effects of BA on YY1 promoter activity. In MDA-MB-453 cells transfected with YY1 p-277 Luc or p-1729 Luc, two luciferase
Figure 24. Effects of BA on Sp1, Sp3, Sp4, ErbB2 and ErbB2 dependent proteins. BA decreases Sp protein and survivin levels in BT474 and MDA-MB-453 cells (A). Cells were treated with 10 µM BA alone or in combination with 1 µM lactacystin for 48 hr, and whole cell lysates were analyzed by western blots as described in the Materials and Methods. B. BA decreases mRNA levels of Sp proteins. Cells were treated with 10 µM BA for 16 hr, and mRNA levels were determined as described in the Materials and Methods. Results are expressed as means ± SE for 3 replicate determinations for each treatment group and significant (p < 0.05) decreases are indicated (*). C. BA decreases protein levels of ErbB2 and ErbB2 dependent proteins. Cells were treated with 10 µM BA alone or in combination with 1 µM lactacystin for 48 hr, and whole cell lysates were analyzed by western blots as described in the Materials and Methods. D. BA decreases YY1 promoter activity. MDA-MB-453 cells were transfected with empty vector (PGL2), the YY1 p-277-luc or the YY1 p-1729-Luc construct, cells were then treated with 5 or 10 µM BA for 24 hr. Luciferase activity was determined as described in the Materials and Methods. Results are means ± SE for 3 separate determinations and significant (p < 0.05) induction of luciferase activity by AP-2 is indicated (*).
reporter constructs containing the -277bp and -1729bp region from the YY1 promoter, treatment with BA for 24 hr resulted in a dose-dependent decrease in luciferase activity (Fig. 24D). RNA interference was used to investigate the potential role of Sp proteins in mediating basal expression of YY1 in BT474 and MDA-MB-453 cells. Figures 25A and 25B illustrate the effect of siRNAs against Sp1 (iSp1), Sp3 (iSp3), Sp4 (iSp4) and their combination (iSp1/3/4) on expression of Sp proteins, YY1 and ErbB2. Transfection of BT474 cells with two siRNAs for Sp1, iSp3, iSp4 and iSp1/3/4 resulted in specific knockdown of the target Sp proteins and Sp1, iSp3, iSp4 and iSp1/3/4 and also decreased expression of YY1 and ErbB2 proteins. In a second set of experiments the oligonucleotides were also transfected in MDA-MB-453 cells (Fig. 25B). The siRNAs for Sp1 and Sp4 proteins were highly specific however iSp3 decreased expression Sp3 and Sp4 proteins; iSp1, iSp4 and iSp1/3/4 decreased levels of both YY1 and ErbB2 whereas Sp3 knockdown had minimal effects on either YY1 or ErbB2 proteins. Previous RNA interference studies showed that knockdown of YY1 also decreased expression of ErbB2 and in this study we also observed that YY1 knockdown decreased ErbB2 in both cell lines (Fig. 25C).

Role of cannabinoid receptors - BA-induced downregulation of Sp transcription factors was proteasome-independent (Fig. 24) and these effects were also not reversed by ROS inhibitors (data not shown) as previously
Figure 25. Role of Sp proteins in regulating level of YY1 protein. Knock down of Sp1, Sp3, Sp4 or Sp1 / Sp3 / Sp4 in combination decreases protein levels of YY1 and ErbB2 in BT474 (A) and MDA-MB-453 (B) cells. Cells were transfected with siRNAs for Sp1, Sp3, Sp4 or Sp1 / Sp3 / Sp4 in combination for 72 hr, and whole cell lysates were analyzed by western blots as described in the Materials and Methods. C. Knock down of YY1 decreases protein levels of ErbB2 in BT474 and MDA-MB-453 cells. Cells were transfected with siRNA for YY1 for 72 hr, and whole cell lysates were analyzed by western blots as described in the Materials and Methods.
reported for CDDO-Me [519]. Since preliminary studies in other cancer cell lines show that cannabinoids (CBs) decrease Sp proteins (data not shown), the effects of CB1 and CB2 receptor antagonists AM251 and AM630 respectively and capsazepine (vanilloid receptor antagonists) on BA-mediated repression of Sp1, Sp3 and Sp4 and survivin were also determined in BT474 and MDA-MB-453 cells (Figs. 26A and 26B). In BT474 and MDA-MB-453 cells, cotreatment of BA with either AM251 or AM630 attenuated the effects of BA downregulation of Sp1, Sp3 and Sp4 whereas capsazepine inhibited the effects of BA only in MDA-MB-453 cells. The effects of AM251, AM630 and capsazepine on BA-mediated downregulation of ErbB2 and ErbB2-regulated kinases were also determined in BT474 (Fig. 26C) and MDA-MB-453 (Fig. 26D) cells. In BT474 cells, the CB1 and CB2 receptor antagonists inhibited BA-mediated downregulation of ErbB2, P-ErbB2, p-MAPK, p-Akt and YY1; similar results were observed in MDA-MB-453 cells confirming a role for the cannabinoid receptors in mediating the effects of BA on Sp and Sp-regulated genes. Treatment of these cells with AM251 or AM630 alone does not affect levels of these proteins (Fig. 26B). Moreover, in MDA-MB-453 cells capsazepine was also active as an inhibitor. Both the CB1 and CB2 receptors are expressed in BT474 and MDA-MB-453 cells (Fig. 26D) however, competitive binding of BA to the CB1 and CB2 receptors using the non-selective radioligand [3H] CP-55, 940 showed that BA at concentrations up to 10 µM did not displace the radioligand. Enhanced binding of [3H] CP-55,940
Figure 26. Role of cannabinoid receptors on effects of BA. Effects of AM251, AM630 and capsazepine on BA-mediated repression of Sp5 and survivin proteins in BT474 (A) and MDA-MB-453 (B) cells. Effects of AM251, AM630 and capsazepine on BA-mediated downregulation of ErbB2 and ErbB2-regulated kinases in BT474 (C) and MDA-MB-453 (D) cells and expression of CB receptors (D). Cells were pretreated with or without 6 μM AM251, 6 μM AM630 or 2 μM capsazepine for 1 hour, and then DMSO or 10 μM BA were added to the medium for 48 hr, and whole cell lysates were analyzed by western blots as described in the Materials and Methods.
to both receptors was observed after coincubation with 100 µM BA and since similar effects were observed for both CB receptors, this may be due to non-specific interactions (Fig. 27A). As a positive control the synthetic cannabinoid WIN-55,212-2 displaced the radioligand from both the CB1 and CB2 receptors (Fig. 27B). Since potential BA-mediated inhibition of fatty acid amide hydrolase (FAAH) would increase production of endocannabinoids to produce a CB1/CB2-dependent downregulation of Sp transcription factors, we investigated the effects of FAAH knockdown by RNA interference on expression of Sp proteins and ErbB2 (Fig 27C). The results show that FAAH knockdown does not affect expression of Sp1 or ErbB2, suggesting that the effects of BA are not due to elevated levels of endocannabinoids acting at CB1 or CB2 receptors resulting from direct inhibition of FAAH.

Previous studies showed that drug-induced repression of Sp1, Sp3 and Sp4 mRNA levels was due to downregulation of miR-27a and induction of ZBTB10, a transcriptional repressor of Sp1, Sp3 and Sp4 gene expression [478,514,519]. Treatment of BT474 and MDA-MB-453 cells with 5 or 10 µM BA resulted in significant downregulation of miR-27a in both cell lines and cotreatment with AM251 or AM630 inhibited this response (Fig. 28A) which was most pronounced in BT474 cells. Downregulation of miR-27a in cells treated with BA was accompanied by induction of ZBTB10 mRNA levels in both cell lines and cotreatment with AM251 or AM630 inhibited the induction response (Fig. 28B). A > 6-fold induction of ZBTB10 was observed in BT474 cells whereas
Figure 27. Supplemental figure for chapter 3. Competitive binding of BA (A) and WIN-55,212-2 (B) to the CB1 and CB2 receptors. C. Effects of FAAH knockdown by RNA interference and the FAAH inhibitor CAY10401 on expression of Sp proteins and ErbB2. Cells were transfected with FAAH siRNA or treated with CAY10401 for 48 hr and western blots were performed as described in Materials and Methods.
Figure 28. Effects of BA on miR-27a and ZBTB10, and the role of cannabinoid receptors on BA-mediated effects. Downregulation of miR-27a (A) and induction of ZBTB10 (B) mRNA levels with BA treatment in BT474 and MDA-MB-453 cells. Cells were pretreated with or without 6 μM AM251 or 6 μM AM630 for 1 hour, and then DMSO 5 μM or 10 μM BA were added to the medium for 24 hr. Total RNA was extracted and miR-27a and ZBTB10 RNA levels were determined as described in the Materials and Methods. Results are expressed as means ± SE for 3 replicate determinations for each treatment group and significant (p < 0.05) decreases or inductions are indicated (*). C. Effects of ZBTB10 overexpression and antisense miR-27a on Sp protein levels, YY1 and ErbB2 proteins. Cells were transfected with 1 μg/ml pCMV6-XL4-ZBTB10 plasmid or empty vector, 50 nM antisense miR-27a (as-miR-27a) or control, and whole cell lysates were analyzed by western blots as described in the Materials and Methods. D. Effects of miR-27a mimic or as-miR-27a on luciferase activity in ZBTB10 3’UTR-luc construct transfected cells. miR-27a mimic (50 nM) or as-miR-27a were transfected into BT474 and MDA-MB-453 cells as described in the Materials and Methods, and a dual luciferase reporter assay was performed according to the manufacturer’s instructions. Results are expressed as means ± SE for 3 replicate determinations for each treatment group and significant (p < 0.05) decreases or inductions are indicated (*).
ZBTB10 was induced >2.5-fold in MDA-MB-453 cells treated with 5 or 10 µM BA. The effects of antisense-miR-27a (as-miR-27a) and ZBTB10 overexpression on levels of Sp1, Sp3 and Sp4, YY1 and ErbB2 proteins were also determined in BT474 and MDA-MB-453 cells (Figs. 28C and 28D) and both treatments decreased expression of Sp and Sp-regulated gene products. The effects of a miR-27a mimic and as-miR-27a on luciferase activity in BT474 and MDA-MB-453 cells transfected with ZBTB10 (UTR)-luc construct containing a miR-27a binding site resulted in decreased (miR-27a mimic) and increased (as-miR-27a) luciferase activity. In contrast, the mimic or antisense oligonucleotide did not affect luciferase activity in cells transfected with a construct [ZBTB10 (mUTR)-luc] containing a mutation in the miR-27a binding sites, confirming interactions of miR-27a with the target sequence in the 3’-UTR of ZBTB10.

The in vivo effects of BA on tumor growth were also investigated in athymic nude mice bearing BT474 cells as xenografts. BA was administered over a period of 28 days at a dose of 20 mg/kg/d and tumor volumes and tumor weight were significantly inhibited and BA also decreased expression of Sp1, Sp3 and Sp4 proteins in tumors (Figs. 29A and 29B). Figure 29C illustrates that immunostaining of ErbB2 and Sp1 proteins were also decreased in fixed tumor tissue from BA-treated mice compared to control (corn oil) treat animals and these in vivo data complement the results of in vitro studies.
Figure 29. BA inhibits tumor (BT474 xenografts) growth. Inhibition of tumor size (A) and weight (B). Athymic nude mice bearing BT474 cells as xenografts were treated with BA (20 mg/kg/d) and tumor sizes and weights were determined as described in the Materials and Methods. Significantly (p < 0.05) decreased tumor sizes and weights are indicated (*). C. BA decreases expression of Sp1, Sp3 and Sp4 proteins in tumors. Whole cell lysates from corn oil and BA-treated tumors were analyzed by western blot as described in Materials and Methods. D, immunostaining for ErbB2 and Sp1. Fixed tumor tissue from corn oil and BA-treated mice were stained with ErbB2 and Sp1 antibodies as described in Materials and Methods.
3.4 Discussion

Endocrine therapies with antiestrogens and aromatase inhibitors for women with early stage ER-positive breast cancer has been one of the most successful cancer treatments in terms of patient prognosis and survival [481,482,485]. In contrast, patients with ER-negative or ErbB2-overexpressing tumors must undergo more aggressive treatment and their overall prognosis and survival are much lower than patients with early stage breast cancer [486-489]. The development of more mechanism-based drugs such as Herceptin for the treatment of ErbB2 overexpressing breast tumors has provided some treatment benefits for a sub-set of patients with these aggressive tumors [491-494]. Herceptin is a monoclonal antibody that binds the extracellular domain of ErbB2 to decrease the tyrosine kinase activity and this results in inhibition of downstream phosphatidylinositol-3-kinase-and mitogen activated protein kinase-dependent phosphorylation of Akt and MAPK respectively [491-494]. Although Herceptin has been successfully used alone and in combination therapy, there is concern regarding cardiotoxic side-effects of this antibody and development of other agents including tyrosine kinase inhibitors is being actively pursued for treatment of tumors which overexpress ErbB2 and other growth factor receptors [526].

TA is an NSAID that has been used for treatment of inflammatory conditions in humans and veterinary medicine [527] and in pancreatic cancer cells and tumors TA inhibited growth and metastasis and induced proteasome-
dependent degradation of Sp1, Sp3, Sp4 and Sp-regulated genes such as VEGF [413,496]. TA also exhibited similar responses in other cells and tumors [515] however, in ErbB2-overexpressing breast cancer cells, the role of Sp protein downregulation in mediating the anticancer activity of TA was cell context-dependent [528]. This was particularly true in BT474 cells where TA inhibited cell and tumor growth but this was accompanied by only 20-30% repression of Sp1, Sp3 and Sp4. Therefore, in order to determine the effects of drug-induced Sp downregulation on ErbB2 overexpressing breast cancer cells and tumors we investigated the effects of BA in BT474 and MDA-MB-453 cells since previous studies with this compound showed that BA inhibited proliferation and induced proteasome-dependent degradation of Sp1, Sp3 and Sp4 in prostate cancer cells [448].

BA also inhibited growth and induced apoptosis in both breast cancer cell lines (Fig. 23) and this was accompanied by downregulation of Sp1, Sp3 and Sp4 protein and mRNA levels (Fig. 24). Moreover, BA also inhibited tumor growth and downregulated Sp1, Sp4 and Sp in tumors from athymic nude mice bearing BT474 cells as xenografts (Fig. 29). The effects of BA on Sp proteins in BT474 and MDA-MB-453 cells were not inhibited after cotreatment with the proteasome inhibitor lactacystin (Fig. 24A) and this was in contrast to the proteasome-dependent degradation of Sp proteins in LNCaP prostate cancer cells treated with BA [448]. In ongoing studies with BA, and other compounds that repress Sp proteins we have observed different mechanisms that are cell
context-dependent [448]. For example, BA decreases Sp proteins in bladder cancer cells and these effects were proteasome-independent [476]. In BT474 and MDA-MB-453 breast cancer cells BA decreased expression of ErbB2 p-ErbB2 and downstream ErbB2-dependent kinases p-MAPK/MAPK and p-Akt/Akt (Fig. 25) suggesting that ErbB2 downregulation may also be due to repression of Sp proteins. However, unlike the EGFR which is an Sp-regulated gene [476], ErbB2 expression is dependent on other transcription factors including YY1 which contains multiple GC-rich promoter sites that bind Sp proteins [525]. BA decreased YY1 protein expression in BT474 and MDA-MB-453 cells (Figs. 25A and 3B) and not surprisingly knockdown of Sp proteins (individually and combined) decreased expression of YY1 and ErbB2 (Figs. 25C and 25D). Moreover YY1 knockdown by RNA interference also decreased ErbB2 (Fig. 25D) demonstrating that BA-mediated downregulation of ErbB2 is linked to decreased expression of Sp1, Sp3, Sp4 and Sp-regulated YY1. Previous studies with TA in BT474 cells and BA in bladder cancer cells show that both compounds repress total Akt and MAPK protein levels [476,528]; similar effects were observed in this study and the mechanisms associated with these responses are currently being investigated.

Previous studies with CDDO-Me in pancreatic cancer cells and CDODA-Me in colon cancer cells showed that repression of Sp mRNA and proteins was due to downregulation of miR-27a in pancreatic, colon and breast cancer cell lines [478,514,519]. Mir-27a regulates expression of ZBTB10, an Sp-repressor,
and BA-dependent repression of miR-27a results in the induction of ZBTB10 [478,514,519]. Results summarized in Figure 27 show that BA also decreased miR-27a and induced ZBTB10 in BT474 and MDA-MB-453 cells suggesting that the critical downstream effects of BA on the miR-27a:ZBTB10 complex are similar to those observed in previous studies with the synthetic triterpenoids CDDO-Me and CDODA-Me [514,519]. CDDO-Me mediated downregulation of miR-27 in pancreatic cancer cells was dependent on upstream disruption of mitochondria and induction of ROS however, in contrast to these results BA did not induce ROS in BT474 and MDA-MB-453 cells and the antioxidant glutathione did not modulate effects of BA on Sp1, Sp3, Sp4 (data not shown).

Ongoing studies in this laboratory with cannabinoids show that these compounds also downregulate Sp1, Sp3 and Sp4 in cancer cell lines (data not shown) and these effects were inhibited after cotreatment with CB1 and CB2 receptor antagonists AM251 and AM630 respectively. Results in Figures 26 and 28 show that AM251 and AM630 inhibited BA-induced miR-27a (downregulation), ZBTB10 (induction), Sp1, Sp3, Sp4, YY1 and ErbB2 (downregulation) and confirm that this pathway involves initial BA-dependent activation of CB1 and CB2 receptors by BA. These results demonstrate for the first time that the effects of BA on BT474 and MDA-MB-453 cells on Sp transcription factors and ErbB2 are mediated through activation of the CB1 and CB2 receptors which subsequently modulate the miR-27a:ZBTB10-Sp axis. CB1 and CB2 receptors are expressed in both cell lines and these receptors are
expressed in human breast cancer cells and tumors and one study showed a
correlation between CB2 receptor and ErbB2 expression in human mammary
tumors [529]. However, since BA does not bind the CB1 and CB2 receptors
(Figs. 27A, 27B) and since inhibition of FAAH does not affect expression of Sp
proteins or ErbB2, the mechanisms associated with activation of CB receptors
by BA are unknown and are currently being investigated.

In summary, this study demonstrates that BA inhibits ErbB2-
overexpressing breast cancer cell and tumor growth and this accompanied a
cascade of events involving activation of the CB1 and CB2 receptors. This
results in modulation of the miR-27a:ZBTB10-Sp transcript-factor axis,
downregulation of the Sp-dependent gene YY1 and the YY1-dependent gene
ErbB2. This pathway does not necessarily define all the anticancer activities of
BA but significantly contributes to the effects of this compound as an inhibitor of
ErbB2-overexpressing breast cancer cell and tumor growth. Current studies are
focused on the mechanistic link between activation of the CB receptors and
modulation of miR-27a:ZBTB10 and the efficacies and mechanism of action of
other agents that repress Sp transcription factors. These data demonstrate a
novel pathway for targeting ErbB2 and suggest new therapeutic approaches for
treating breast cancer patients that overexpress this oncogene.
4. MECHANISM OF ACTION OF ASPIRIN AS AN ANTICANCER AGENT IN BREAST CANCER

4.1 Introduction

Acetylsalicylic acid (ASA) or aspirin is a non-steroidal anti-inflammatory drug (NSAID) widely used for treatment of pain, fever and other inflammatory conditions [530] and the role of ASA and other NSAIDs in cancer has been extensively investigated [417,531-533]. There is evidence for the association of ASA use and decreased risk for colorectal, breast, esophageal, lung, stomach and ovarian cancer, whereas effects on the incidence of other cancers are uncertain [417,531-533]. Several studies consistently show that aspirin is a chemopreventive agent for breast and colon cancer [534-541] and there is also evidence that aspirin exhibits remarkable chemotherapeutic activity for these cancers. For example, a hazard ratio of 0.53 (for mortality) was observed in colorectal cancer patients who did not use the drug prior to diagnosis and this value decreased to 0.39 for a subset of patients overexpressing cyclooxygenase-2 (COX-2) [541]. Several studies have demonstrated the protective effects of aspirin in breast cancer incidence [542-550]. A recent report on breast cancer patients from the prospective Nurses’ Health Study showed that the adjusted relative risks for breast cancer patients using aspirin 1, 2-5 or 6-7 days/wk were 0.91, 0.57 and 0.40, respectively [550]. These dramatic chemotherapeutic effects of aspirin for colorectal and breast cancer demonstrate
the potent anticancer activity of this drug and the potential chemotherapeutic
efficacy for applications of aspirin in drug combinations.

The effects of aspirin on cancer cells in culture and in in vivo models
demonstrate the anticancer activity of this compound in multiple tumor types and
there is evidence for induction of apoptosis, inhibition of cell proliferation and
angiogenesis [435,551-559]. These aspirin-induced responses are somewhat
variable among cancer cell types and have been linked to decreased expression
or activity of NFkB, bcl-2, vascular endothelial growth factor (VEGF) and Wnt/β-
catenin.

Research in this laboratory has been focused on development of anticancer
agents that downregulate specificity protein (Sp) transcription factors Sp1, Sp3
and Sp4 that are overexpressed in many tumor types. Several different classes
of compounds repress Sp transcription factors and Sp-regulated genes and
these include betulinic acid and synthetic triterpenoid analogs, curcumin, arsenic
trioxide non-steroidal antiinflammatory drugs (NSAIDs) and a nitro-NSAID
derivative [407,413,448,475-477,496,514,515,519,520,528,560-563]. The
rationale for targeting Sp proteins is due to the importance of Sp-regulated gene
products many of which are individually targeted by tyrosine kinase inhibitors or
neutralizing antibodies. Sp-regulated genes that are decreased by anticancer
drugs and by RNA interference (Sp knockdown) include epidermal growth factor
receptor (EGFR), hepatocyte growth factor receptor (c-MET) survivin, bcl-2,
cyclin D1, VEGF and its receptors (VEGFR1/R2) and pituitary tumor
transforming gene-1 (PTTG-1) [369,448,475-477,518-520]. Recent studies show that the NO-NSAID ethyl 2-[(2,3-bis (nitrooxy) propy] disulfanyl benzoate (GT-094) inhibits colon cancer cell growth and this is due, in part, to downregulation of Sp1, Sp3, Sp4 and Sp-regulated genes [562] and in this report we investigated the effects of aspirin on ErbB2-overexpressing BT474 and MDA-MB-453 breast cancer cells. The results show the aspirin decreased expression of Sp1, Sp3, Sp4 and several Sp-regulated genes including YY1 and this latter response resulted in decreased expression of ErbB2 which is regulated by YY1.

4.2 Materials and methods

Chemicals, antibodies and reagents - Aspirin, camptothecin, lactacystin, phosphatase inhibitor cocktail II, sodium orthovanadate and MKP-5 (D7944) antibody were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against ErbB2 (C-18), p-ErbB2 (Try 1248)-R, Sp1 (PEP2), Sp3 (D-20), Sp4 (V-20), Akt (H-136), p-Akt (Ser473), MAPK(C-14), p-MAPK (E-4), β-actin (C4), β-tubulin (H-235), VEGF (A20), AP-2α (C-18) and YY1 (H-10) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); Antibodies against cleaved-PARP (D214) and survivin were obtained from Cell Signaling Technology (Danvers, MA). The negative control siRNA (4613) siRNA for MKP-5 and MKP-1 was purchased from Ambion (Austin, TX). Western lightning chemiluminescence reagent was from Perkin-Elmer Life Sciences (Boston, MA). Lipofectamin 2000 was purchased from Invitrogen.
Cell lines - Human mammary carcinoma cell lines MDA-MB-453, and BT474 were obtained from the American Type Culture Collection (Manassas, VA). Cell lines were cultured with 10% fetal bovine serum (FBS) in DMEM and were maintained at 37°C in the presence of 5% CO₂.

Cell proliferation assay - Cells (2-3 x 10⁴ per well) were plated in 12-well plates and allowed to attach for 24 hr. Then cells were treated with either vehicle (DMSO) or different concentrations of aspirin for 48 hr, and cells were then trypsinized and counted using a Coulter Z1 cell counter. Each experiment was done in triplicate, and results are expressed as means ± SE for each set of experiments.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling - Cells were plated in Lab-Tek II Chamber Slide System (Nalge Nunc International, Naperville, IL) and allowed to attach for 24 hr. Then cells were treated with either vehicle (DMSO) or different concentrations of aspirin for 24 hr, TUNEL staining was performed using the DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI) following manufacture’s protocol. The slides were analyzed using a Nikon ECLIPSE 80i confocal microscope (Japan).

Cell migration assay - Cells were seeded 70% confluent in triplicates in six-well collagen IV–coated plates and then treated with selected concentration of aspirin for 24 hours, then a scratch through the central axis of the plate was gently made using a sterile 200 ul pipette tip. Cells were then washed with medium and treated with the DMSO control or different concentration of aspirin.
Migration of the cells into the scratch was observed at nine preselected points (three points per well) at 0, 24, and 48 hour. Results of this study were obtained at a 48 hour time point.

**Western blotting** - Cells were rinsed with PBS and collected by scraping cells from the culture plate in 200 μL of lysis buffer. The cell lysates were incubated on ice for 1 hr with intermittent vortex mixing and then centrifuged at 40,000 g for 10 min at 4°C. Equal amounts of protein were separated on SDS-polyacrylamide gels. Proteins were transferred to Immobilon P membranes (Millipore, Bedford, MA) using a Bio-Rad Trans-blot apparatus and transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS, 20% methanol). After blocking in TBST-Blotto [10 mmol/L Tris-HCl, 150 mmol/L NaCl (pH 8), 0.05% Triton X-100, 5% nonfat dry milk] for 30 min, the membranes were incubated with primary antibodies overnight at 4°C and then with horseradish peroxidase-conjugated secondary antibody for 2 hr at room temperature. Proteins were visualized using the chemiluminescence substrate (Perkin-Elmer Life Sciences) for 1 min and exposed to Kodak X-OMAT AR autoradiography film (Eastman Kodak, Rochester, NY).

**Quantitative real-time PCR** - Total RNA was purified using RNeasy Mini Kit (Qiagen, Germantown, MD) and cDNA was prepared using Reverse Transcription System (Promega, Madison, WI). Each PCR was carried out in triplicate in a 30 μL volume using SYBR Green Mastermix (Applied Biosystems, Foster City, CA) for 15 min at 95°C for initial denaturing, followed by 40 cycles of
95°C for 30 s and 60°C for 1 min in the Applied Biosystems 7900HT Fast Real-time PCR System. The ABI Dissociation Curves software was used following a brief thermal protocol (95°C for 15 s and 60°C for 15 s, followed by a slow ramp to 95°C) to control for multiple species in each PCR amplification. Values for each gene were normalized to expression levels of TATA-binding protein (TBP). Primers were obtained from Integrated DNA Technologies. The following primers were used:

MKP-5(F): 5'-ATC TTG CCC TTC CTG TTC CT-3'.
MKP-5(R): 5'-ATT GGT CGT TTG CCT TTG AC-3'.
MKP-1(F): 5'-CTC CAT GCT CCT TGA GAG GAG-3'.
MKP-1(R): 5'-GGT AGG TAT GTC AAG CAT GAA-3'.
FAP-1(F): 5'-GAA TAC GAG TGT CAG ACA TG-3'.
FAP-1(R): 5'-AGG TCT GCA GAG AAG CAA GA-3'.
PTP-1B(F): 5'-ACG AAT CCT GGA GCC ACA CAA TG-3'.
PTP-1B(R): 5'-AGC CCA CGC ACA TGT TGA CCA GGA-3'.
Se-PAP(F): 5'-GGC AGA TGA TGC TTT GAG AAC-3'.
Se-PAP(R): 5'-TCA TCC AAA GCC CAT TTT CC-3'.
TM-PAP(F): 5'-TCT CAG TGG TGC CGC ATC TA-3'.
TM-PAP(R): 5'-CAG GGT GTG AGG ATG GCA A-3'.
TBP (F): 5'-TGC ACA GGA GCC AAG AGT GAA-3'.
TBP (R): 5'-CAC ATC ACA GCT CCC CAC CA-3'.
siRNA transfection - Cells at 50% confluency in 60 mm collagen-coated dishes were transfected with 50 nmol of negative control or MKP-5 / MKP-1 specific siRNA using Lipofectamin 2000. Transfection with siRNA was accomplished according to the manufacturer's instructions. Transfection reagent was not removed from the cells and transfected cells were incubated for 72 hours at 37°C before experimentation.

4.3 Results

BT474 AND MDA-MB-453 cells were treated with 1 – 10 mM aspirin for 48 hr.; cell growth was inhibited by 1.5 and 10 mM aspirin and IC<sub>50</sub> values were 4.1 and 2.13 mM respectively (Fig. 30A). Similar results were observed in estrogen receptor (ER)-negative SKBR3 and MDA-MB-231 cells (data not shown). MDA-MB-453 and BT474 cells were also treated with DMSO (solvent control), 2.5 and 5.0 mM aspirin for 24 hr. and analyzed for apoptosis in a TUNEL assay (Fig. 30B). After staining with DAPI and FITC there was increased TUNEL staining in both cells and similar results were observed after treatment of BT474 cells with 0.2 ug/ml camptothecin which served as a positive control for this experiment (Fig. 30B). The effects of 1 or 2.5 mM aspirin of cell migration were also investigated in MDA-MB-453 and BT474 cells (Fig. 30C). Treatment for 48 hr. inhibited cell growth in both cell lines; however migration was inhibited only in MDA-MB-453 cells. Under the conditions used in this experiment neither cell line exhibited high levels of migration and BT474 cells did not significantly migrate in the solvent (DMSO) control group.
Figure 30. Effects of aspirin on cell proliferation, apoptosis and migration. A. Aspirin-mediated inhibition of BT474 and MDA-MB-453 cell growth. Cells were treated with solvent (DMSO) or different concentrations of aspirin for 2 days and the number of cells in each treatment group were determined as described in the Materials and Methods. Cell survival is expressed as the percentage of aspirin-treated cells remaining compared to DMSO (set at 1). Significant (p < 0.05) growth inhibition is indicated (*). Results are expressed as means ± SE for at least 3 replicate determinations for each treatment group.

B. Aspirin induces apoptosis in cancer cells. Cells were treated with DMSO, 2.5, 5.0 mM aspirin or 0.2 ug / ml camptothecin for 24 hours and analyzed with TUNEL as described in the Materials and Methods.

C. Cell migration assay. MDA-MB-453 and BT474 cells were treated with DMSO, 1 mM or 2.5 mM aspirin for 48 hours, migration of cells were determined as described in the Materials and Methods.
Figure 31A shows that aspirin-induced growth inhibition and apoptosis observed in BT474 cells (Fig. 30) is accompanied by induction of cleaved PARP and downregulation of the survival protein survivin and VEGF (Fig. 31A). Moreover, aspirin also decreased phosphorylation of ErbB2 which is a critical growth/survival-promoting factor in BT474 cells. However, this was also accompanied by decreased expression of ErbB2 (total) protein (Fig. 31C). We also observed decreased phosphorylation of AKT and MAPK and this was also accompanied by decreased expression of total AKT protein but much less pronounced changes in total MAPK protein were observed. In a parallel set of experiments in MDA-MB-453 cells, treatment with aspirin also decreased expression of survivin and VEGF and induced PARP cleavage (Fig. 31B) and the effects on phospho- and total ErbB2, AKT and MAPK proteins (Fig. 31D) were similar to those observed in BT474 cells.

Results of ongoing and published studies from this laboratory show that downregulation of Sp1, Sp3 and Sp4 proteins by various agents or by RNA interference can lead to the changes in protein expression observed in Figure 31 [448,475-477,514,515,519,520] and therefore the effects of aspirin on Sp1, Sp3 and Sp4 expression were investigated. Aspirin induced a concentration- and time-dependent decrease in Sp1, Sp3 and Sp4 protein levels in BT474 and MDA-MB-453 cells (Fig. 32A). Some agents that induce downregulation of Sp proteins activate the proteasome pathway [448,475] and results in Figure 32B
Figure 31. Effects of aspirin on survival proteins, cleaved-PARP, ErbB2 and ErbB2-dependent proteins. VEGF, survivin and c-PARP expression in BT474 (A) and MDA-MB-453 (B) cells treated with aspirin. Cells were treated with different concentrations of aspirin for 24 or 48 hours and whole cell lysates were analyzed by western blots as described in the Materials and Methods. ErbB2 and ErbB2-dependent protein expression in BT474 (C) and MDA-MB-453 (D) cells treated with aspirin. Cells were treated as described in (A), (B) and whole cell lysates were analyzed by western blots as described in the Materials and Methods.
show that aspirin-induced downregulation of Sp1, Sp3 and Sp4 proteins in BT474 and MDA-MB-231 cells was partially reversed after cotreatment with the proteasome inhibitor lactacystin. These results were obtained after treatment of the cells with 2.5 mM aspirin and 1 μM lactacystin for 48 hr. and higher concentrations of either compound were cytotoxic. Thus aspirin-mediated downregulation of Sp1, Sp3 and Sp4 was due in part to activation of the proteasome pathway as previously observed for the NSAID tolfenamic acid and betulinic acid in pancreatic and prostate cancer cells respectively [448,475].

YY1 regulates ErbB2 expression in BT474 [319] and MDA-MB-453 [564] and recent studies in this laboratory have demonstrated by RNA interference that YY1 is also an Sp-regulated gene in both cell lines [564]. Figures 32C and 32D show that aspirin also decreased YY expression in both cell lines and this effect is reversed in cells cotreated with aspirin plus the proteasome inhibitor lactacystin. AP2 is a transcription activator of ErbB2. Therefore expression of AP2 was also investigated. AP2 expression was not changed in BT474 and MDA-MB-453 cells treated with aspirin for 24 hr and a slight decrease was observed when cells are treated with 5 mM aspirin for 48 hr (Fig. 32C); and this effect was not reversible in cells cotreated with aspirin plus lactacystin (Fig. 32D).

Phosphorylation of Sp1 increases Sp-1 dependent transactivation[565] and also protects against proteasome-dependent degradation of Sp1 [347] suggesting that aspirin-induced proteasome-dependent degradation of Sp1, Sp3
Figure 32. Effects of aspirin on Sp, YY1 and AP2 proteins. Sp1, Sp3 and Sp4 expression in BT474 and MDA-MB-453 cells treated with aspirin alone (A) or in combination with lactacystin (B). Cells were treated with different concentrations of aspirin (A) or pretreated with 1 uM lactacystin for 1 hour followed by addition of aspirin (B) for 48 hours and whole cell lysates were analyzed by western blots as described in the Materials and Methods. YY1 and AP2 expression in BT474 and MDA-MB-453 cells treated with aspirin alone (C) or in combination with lactacystin (D). Cells were treated as described in (A), (B) and whole cell lysates were analyzed by western blots as described in the Materials and Methods.
and Sp4 may be due to induction of phosphatases. Results in Figure 33A show that PICII, a commercial mixture of phosphatase inhibitors (sodium orthovanadate, sodium molybdate, sodium tartrate and imidazole) inhibited aspirin-induced downregulation of Sp1, Sp3 and Sp4 in BT474 cells whereas another mixture of phosphatase inhibitors (PIC I) had minimal effects (data not shown). Results of preliminary screening of PICII components indicated that sodium orthovanadate (SOV) was the most active inhibitor (Fig. 33A) and the aspirin-induced effects on Sp proteins were inhibited by PICII and SOV in BT474 cells. In MDA-MB-453 cells aspirin-induced downregulation of Sp1, Sp3 and Sp4 were also inhibited by PICII and SOV (Fig. 33B). SOV was used as an inhibitor of aspirin-induced downregulation of survivin and induction of PARP cleavage in BT474 and MDA-MB-453 cells and the effects of aspirin were reversed by SOV (Fig. 33C). Moreover, SOV also inhibited aspirin-induced downregulation of ErbB2 (total) and phosphorylated ErbB2, AKT and MAPK (Fig. 33D) and the effects of aspirin illustrated in Figures 33C and 33D were also inhibited by PICII (data not shown).

Several different classes of anticancer agents including curcumin, a compound that also decreases expression of Sp1, Sp3 and Sp4 in cancer cells [475,477] also induce phosphatase gene expression [566-571] and Figures 34A and 34B summarize the effects of aspirin on induction of 6 phosphatases in BT474 and MDA-MB-453 cells respectively. Like curcumin [566], aspirin induces MKP-5 and also MKP-1 in both cell lines; soluble prostatic acid phosphatase (s-
Figure 33. PIC II or Na3VO4 reverses the effects of aspirin on Sp, survival proteins, ErbB2 and ErbB2-dependent proteins. Expression of Sp1, Sp3 and Sp4 proteins in BT474 (A) and MDA-MB-453 (B) cells treated with aspirin with or without PIC II or Na3VO4. Cells were pretreated with or without PIC II or Na3VO4 for 1 hour followed by addition of different concentrations of aspirin for 48 hours and whole cell lysates were analyzed by western blots as described in the Materials and Methods. Expression of survivin, c-PARP (C), ErbB2 and ErbB2-dependent (D) proteins in BT474 and MDA-MB-453 cells treated with aspirin with or without 5 mM Na3VO4. Cells were pretreated with or without Na3VO4 followed by addition of aspirin as described in (A, B) and whole cell lysates were analyzed by western blots as described in the Materials and Methods.
Figure 34. Effects of aspirin on phosphatases. Effects of aspirin on MKP-5, MKP-1, Se-PAP, TM-PAP, FAP-1 and PTP-1B mRNA levels in BT474 (A) and MDA-MB-453 (B) cells. Cells were treated with 5 mM aspirin for up to 16 hr (a), or treated with different concentration of aspirin for 24 hr (b), and phosphatase mRNA levels were determined by real time PCR as described in the Materials and Methods. Results are expressed as means ± SE for 3 replicate determinations for each treatment group and significant (p < 0.05) inductions are indicated (*). Roles of MKP-5 (C) and MKP-1 (D) in aspirin-induced downregulation of Sp proteins, ErbB2 and YY1. Cells were transfected with control siRNA, siRNAs for MKP-5 or MKP-1, then DMSO or 5 mM aspirin was added to the medium and cells were incubated in 37 °C for 48 hr. Whole cell lysates were analyzed by western blots as described in the Materials and Methods.
Figure 34. (Continued)
PAP) was not induced by aspirin and cellular PAP (c-PAP) was induced in MDA-MB-453 but not BT474 cells. Two additional phosphatases (FAP-1 and PTP-1B) were not induced in either cell lines. Since aspirin and curcumin decrease expression of Sp1, Sp3, and Sp4 and Sp-regulated gene products (Figs. 31-32) and also induce MKP-5 mRNA we hypothesized that MKP-5-dependent dephosphorylation of Sp proteins may be important for activation of the proteasome pathway. Results in Figure 34C demonstrate that aspirin-induced downregulation of Sp1, Sp3 and Sp4 are accompanied by induction of MKP-5 protein and in cells transfected with an oligonucleotide (siMKP5) that knocks down MKP-5 by RNA interference the effects of aspirin on Sp1, Sp3 and Sp4 are reversed. MKP1 protein level was induced in BT474 but not in MDA-MB-453 cells when treated with aspirin, and knockdown of MKP1 in BT474 cells does not reverse aspirin-induced downregulation of Sp1, Sp3 and Sp4 (Fig. 34D). This demonstrates for the first time a linkage between a drug-induced phosphatase and Sp downregulation and is consistent with studies by Hung, Chang and coworkers showing that phosphorylation stabilizes Sp1 [347].

4.4 Discussion

Sp transcription factors are overexpressed in multiple tumor types and Sp1 is a negative prognostic factor for survival of gastric and colon cancer cell lines [368,572]. Although most Sp transcription factors are critical in embryonic and early postnatal development [573] there is evidence in rodents and humans that Sp1 expression decreases with age [516,517,574] in mouse tumor xenograft
studies expression of Sp1, Sp3 and Sp4 is low in most organs and high in tumors [413, 448, 477]. Lou and coworkers reported that carcinogen-induced transformation fibroblasts that formed tumors in xenograft experiments exhibited an 8- to 18-fold increase in Sp1 expression in the transformed cells [575]. Moreover, knockdown of Sp1 in the transformed cells resulted in the loss of tumor formation of these cells in animal models thus demonstrating the pro-oncogenic activity of Sp1.

The importance of Sp1, Sp3 and Sp4 as targets for anticancer drugs is due primarily to Sp-regulated growth promoting (cyclin D1, p65, c-MET and EGFR), antiapoptotic (survivin and bcl-2) and angiogenic (VEGF, VEGFR1/R2 and PTTG-1) genes. Studies in this laboratory have identified several anticancer agents that induce proteasome-dependent or –independent downregulation of Sp1, Sp3 and Sp4 regulated gene products, and the mechanism of action of these responses are cell context- and compound-dependent. Nevertheless, the development and mechanism of action of compounds such as curcumin, arsenic trioxide, NSAIDs, betulinic acid and synthetic triterpenoids that decrease Sp1, Sp3, Sp4 and Sp-regulated genes is important for the future clinical applications of these drugs alone and in combination therapies.

NSAIDs such as tolfenamic acid target Sp proteins in most but not all cell lines and the NO-NSAID GT-094 also exhibits similar activity in colon cancer cells [413, 496, 561, 562]. Since aspirin exhibits chemotherapeutic and chemopreventive activity in breast cancer patients [542-550] we hypothesized
that this compound may act by targeting Sp transcription in breast cancer cells. Recent studies in this laboratory showed that betulinic acid decreased expression of Sp1, Sp3, Sp4 and Sp-regulated gene products in ErbB2-overexpressing breast cancer cells (BT474 and MDA-MB-453), and it was demonstrated that decreased expression of ErbB2 was due to downregulation of YY1 an Sp-regulated gene that is important for ErbB2 expression [319,564]. Aspirin inhibited BT474 and MDA-MB-453 cell growth and induced apoptosis (Figs. 30A and 30B) at low mM concentrations as reported in other cancer cell lines [319]. Moreover, aspirin decreased expression of Sp1, Sp3 and Sp4 proteins and surviving, and this was accompanied by induction of cleaved PARP in both cell lines as previously described for betulinic acid in these same cell lines [564] and for other agents that target Sp transcription factors.

The growth inhibitory effects of BA in BT474 and MDA-MB-453 cells was due, in part, to downregulation of ErbB2, phospo-ErbB2 and ErbB2-regulated genes [564] and this was also observed for aspirin in these cell lines (Fig. 31). Moreover like BA [564] aspirin also decreased expression of the Sp-regulated gene YY1 (Figs. 32C and 32D) and this is consistent with the downregulation of ErbB2 which is regulated by YY1. The effects of BA in breast cancer cells and GT-094 (NO-NSAID) in colon cancer cells were proteasome independent and involved disruption of microRNA:ZBTB10 interactions resulting in enhanced expression of the transcriptional repressor ZBTB10, an Sp repressor that decreases expression of Sp and Sp-regulated genes [562]. In contrast, the
effects of aspirin on Sp1, Sp3 and Sp4 are due to activation of proteasomes and these effects can be blocked by the proteasome inhibitor lactacystin (Fig. 32B) and this pathway has previously been observed for tolfenamic acid and betulinic acid in pancreatic and colon cancer cells respectively [448,475]. The mechanism of this response has not been determined. However a recent study showed that phosphorylation of Sp1 stabilized this protein and inhibited ubiquitination and proteasome-dependent degradation [347]. Not surprisingly phosphatase inhibitors such as PICII and SOV inhibited aspirin-induced downregulation of Sp1, Sp3, Sp4 and Sp regulated genes (Fig. 33), suggesting a role for phosphatases in mediating decreased expression of Sp proteins.

Previous studies in colon cancer cells show that aspirin inhibited protein phosphatase 2A activity [553]. However it has also been reported that curcumin induced MKP5 in prostate cancer cells [566] and we have previously showed that curcumin decreased Sp transcription factors in bladder and pancreatic cancer cells and tumors [475,477]. Aspirin also induces MKP-5 and other phosphatases in BT474 and MDA-MB-453 cells (Figs. 34A and 34B) and knockdown of MKP-5 inhibited aspirin-induced effects on Sp proteins (Fig. 34C). These results suggest that for aspirin in BT474 and MDA-MB-453 cells, a key event in downregulation of Sp1, Sp3 and Sp4 is associated with induction of MKP-5 and phosphatase activity. These effects may be cell context-specific and current studies are focused on the induction and role of other phosphatases in mediating downregulation of Sp1, Sp3 and Sp4 and on identification of other
factors such as ubiquitin ligases that may also be required for activating proteasomes pathways that primarily target Sp1, Sp3 and Sp4. Our results identify for the first time that the mechanisms of action of aspirin as a chemotherapeutic agent for treatment of breast cancer is due, in part, to activating proteasomes that targeted Sp1, Sp3 and Sp4 transcription factors and this is accompanied by decreased expression of growth promoting, antiapoptotic and angiogenic Sp-regulated genes. These effects include downregulation of YY1 and ErbB2 (a YY1-regulated gene) and illustrate a novel pathway for targeting ErbB2 and also demonstrate why aspirin is a highly effective chemotherapeutic agent for treatment of breast cancer. These results also suggest that other agents that decrease Sp1, Sp3 and Sp4 transcription factors may also be effective for breast cancer chemotherapy, and our ongoing research is focused on characterization and clinical applications of these compounds.
5. SUMMARY

Treatment of patients with ER-positive breast cancer with endocrine therapies such as SERMs and aromatase inhibitors has been highly successful in terms of improving patient survival [481,482,485]. However, approximately 20 to 30 percent of breast cancer patients overexpress ErbB2 and treatment for women with ER-negative or ErbB2-positive breast tumors is relatively more intractable and the overall prognosis and survival of this sub-set of patients are much lower than patients with early stage ER-positive breast cancer [486-489]. The development of Herceptin for treatment of ErbB2 overexpressing breast tumors has brought significant benefits to this subset of patients. Herceptin is a monoclonal antibody that binds to the extracellular domain of ErbB2 to block dimerization which results in increased endocytosis and degradation of ErbB2 resulting in inhibition of downstream activation of PI3K and MAPK signaling. Although Herceptin has been successfully used alone and in combination with other drugs, cardiotoxicity and fetal-toxicity have been reported as side-effects [493,576]. Development of other agents that target ErbB2 include other monoclonal antibodies (Pertuzumab), tyrosine kinase inhibitors (Lapatinib), ansamycin antibiotics (geldanamycin), proteasome inhibitors (bortezomib), and FAS inhibitors [220,270,492-494,497-501,503-508]. Although the mechanisms of action of these compounds are highly variable, all the compounds block activation of ErbB2 and downstream responses.
Sp transcription factors are over expressed in multiple tumor types compared to normal tissues and Sp1 is a negative prognostic factor for survival of gastric and colon cancer patients [368,572]. Sp transcription factors have been investigated as important anticancer drug targets, and this is due in part to regulation of pro-oncogenic factors by Sp transcription factors and these include genes involved in growth promotion (cyclin D1, E2F1, c-fos, p65, c-MET and EGFR) [374-377], cell survival (survivin, bcl-2) [383-385] and angiogenesis (VEGF, VEGFR1 /R2, PTTG-1) [369,382].

TA is a relatively non-toxic NSAID used in humans for treatment of migraines and as an anti-inflammatory drug in veterinary medicine. Development of TA exhibits relatively low toxicity and previous studies reports show that TA inhibited growth and metastasis of pancreatic cancer cells and tumors through inducing proteasome-dependent degradation of Sp1, Sp3, Sp4 and Sp-regulated genes such as VEGF [413,496]. TA also exhibited similar effects in esophageal cancer cells and tumors; however the effects on Sp downregulation were proteasome-independent in these cells [515]. In this study, TA inhibited growth of ErbB2-overexpressing BT474 and SKBR3 breast cancer cells. However, in contrast to pancreatic cancer cells, TA induced downregulation of ErbB2 but not Sp proteins. TA-induced ErbB2 downregulation was accompanied by decreased ErbB2-dependent kinase activities, induction of p27, and decreased expression of cyclin D1. TA also decreased ErbB2 mRNA expression and promoter activity, and this was due to decreased mRNA stability in BT474 cells and, in both cell
lines, TA decreased expression of the YY1 and AP-2 transcription factors required for basal ErbB2 expression. In addition, TA also inhibited tumor growth in athymic nude mice in which BT474 cells were injected into the mammary fat pad. In contrast to mechanisms of other ErbB2-targeting drugs, downregulation of ErbB2 by TA is not due to enhanced ErbB2 degradation, induction of ErbB2 repressors or increased ErbB2 endocytosis (Fig. 35A). Thus, the anticancer activity of TA is associated with downregulation of Sp transcription factors in some cell lines [369,413,496]. However repression of the oncogene ErbB2 in ErbB2-positive breast cancer cell lines was independent of effects on Sp transcription factors. It is possible that the effects of TA on expression of other factors that regulate YY1 and AP2 in BT474 and SKBR3 cells are important for downregulation of ErbB2 and the mechanisms of this response and potential clinical applications of TA for treatment of ErbB2-overexpressing cancers are currently being investigated.

BA is a natural pentacyclic triterpenoid which exhibited selective cytotoxicity against a number of specific tumors, a variety of infectious agents such as HIV, malaria and bacteria, and the inflammatory process [445]. Biological activity of BA was first demonstrated in melanoma cell lines and was confirmed in mice bearing human melanoma cells as xenografts [460]. These in vivo studies demonstrated a favorable safety margin for BA, as systemic side effects were not observed at any dose up to 500 mg per kg body weight [440,460]. Previous studies in this laboratory reported that BA inhibited prolifera-
Figure 35. Model of mechanisms of TA (A), BA (B) and aspirin (C).
tion and induced proteasome-dependent degradation of Sp1, Sp3 and Sp4 in prostate cancer cells [448]. BA also decreases Sp proteins in bladder cancer cells and these effects were proteasome-independent [476]. In this study, treatment of ErbB2 overexpressing BT474 and MDA-MB-453 breast cancer cells with 1-10 μM BA inhibited cell growth and induced apoptosis through downregulation of both Sp proteins and ErbB2; this effect was proteasome-independent as observed for BA in bladder cancer cells but contrasted to BA-induced proteasome-dependent degradation of Sp1, Sp3 and Sp4 in prostate cancer cells [448]. In this study, BA also induced downregulation of survivin, a Sp-regulated gene, ErbB2-regulated kinases, YY1 and luciferase activity in cells transfected with a construct containing the GC-rich YY1 promoter linked to a luciferase reporter gene. Knockdown of Sp1, Sp3, Sp4 and their combination by RNA interference was also accompanied by decreased expression of ErbB2 and YY1. However, unlike the EGFR which is an Sp-regulated gene, ErbB2 expression is dependent on other transcription factors including YY1 which contains multiple GC-rich promoter sequences that bind Sp proteins [525], suggesting indirect regulation of ErbB2 by Sp transcription factors. BA-dependent repression of Sp1, Sp3, Sp4 and Sp regulated genes was due in part to induction of the Sp repressor ZBTB10 and down regulation of microRNA-27a (miR-27a) which constitutively inhibits ZBTB10 expression. The effects of BA on the miR-27a:ZBTB10-Sp transcription factor axis were inhibited in cells cotreated with the cannabinoid 1 (CB1) and CB2 receptor antagonists AM251 and AM630.
respectively (Fig. 35B). However, *in vitro* binding studies with ≤ 10 µM BA and a radiolabeled cannabinoid did not indicate competitive binding of BA to the CB1 and CB2 receptors, suggesting a possible role for other CB-like G protein-coupled receptors. In the athymic nude mice xenograft studies, BA not only inhibited tumor growth but also decreased expression of Sp1, Sp3, Sp4 and ErbB2 in the tumors. Previous studies with TA in BT474 cells and BA in bladder cancer cells show that both compounds repress total Akt and MAPK protein levels [510,511]; similar effects were observed in this study and the mechanisms associated with these responses are currently being investigated.

Aspirin is a widely used NSAID for relieving minor aches, reducing fever and as an anti-inflammatory agent. Although aspirin induces considerable side-effects such as gastrointestinal ulcers, stomach bleeding, and tinnitus in higher doses, prolonged use of aspirin results in chemotherapeutic and chemopreventive activity in breast cancer patients [542-550] and this prompted us to investigate the effects and mechanism of action of aspirin in ErbB2-overexpressing breast cancer cells. Aspirin inhibited growth, induced apoptosis and inhibited migration of BT474 and MDA-MB-453 breast cancer cells that overexpress the ErbB2 oncogene. Aspirin also downregulated ErbB2 expression in these cells and this was accompanied by inhibition of downstream kinases including phospho-Akt and phospho-mitogen-activated protein kinase (p-MAPK). Aspirin also decreased expression of Sp1, Sp3, Sp4 and survivin, and this effect was inhibited in part by proteasome inhibitor lactacystin and phosphatase
inhibitors including sodium orthovanadate (SOV), suggesting a role for proteasomes and phosphatases in aspirin-induced downregulation of Sp proteins (Fig. 35C). These results were consistent with induction of several phosphatases by aspirin in BT474 and MDA-MB-453 cells and these include MKP-5 and MKP-1. Aspirin-induced downregulation of Sp1, Sp3 and Sp4 were reversed in cells transfected with an oligonucleotide (siMKP5) that knocks down MKP5 by RNA interference demonstrating for the first time a linkage between a drug-induced phosphatases and Sp downregulation and this is consistent with studies showing that phosphorylation stabilizes Sp1 [347]. Aspirin-induced induction of MKP-1 mRNA was not accompanied by increased MKP-1 protein level in MDA-MB-453 cells suggesting possible enhanced degradation or repression of translation of MKP-1 in this cell line. Knockdown of MKP-1 in BT474 cells did not affect aspirin-induced downregulation of Sp proteins and ErbB2 implicates that induction of MKP-1 mRNA and protein levels were not accompanied by increased activity of this protein. These results demonstrated that aspirin-induced downregulation of Sp1, Sp3 and Sp4 is due to induction of MKP and the role of MKP2 in destabilizing Sp proteins and facilitating degradation by proteasomes is currently being investigated.

In summary, these studies demonstrated that TA, BA and aspirin inhibited ErbB2-overexpressing breast cancer cell and tumor growth by targeting Sp / ErbB2 through different mechanisms. Results of these studies do not necessarily define all the anticancer activities of TA, BA and aspirin but identify
important pathways that significantly contribute to the effects of these compounds as inhibitors of ErbB2-overexpressing breast cancer cell and tumor growth.
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Name: Xinyi Liu

Address: Texas A&M Health Science Center IBT
         Rm 413, 2121 W Holcombe Blvd
         Houston, TX 77030

Email Address: xinyiliu@tamu.edu

Education: B.MS., Chongqing Medical University
           Chongqing, China, 2001

Ph.D., Biochemistry, Texas A&M University, 2011