Molecular targets in breast cancer: current status and future directions

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Abstract
Our increasing understanding of the pathophysiologic mechanisms of breast carcinogenesis has generated detailed information about the potential roles of specific biomolecular markers in this process. Furthermore, in the last few years the process of targeted drug design has become faster and more sophisticated, providing a variety of agents targeted at these molecules. In this review, we describe the most widely recognized molecular targets in breast cancer.

Introduction
Over the last several years, our understanding of the molecular mechanisms involved in the process of breast carcinogenesis has increased dramatically. Studies have elucidated the specific risk of malignant transformation associated with premalignant breast lesions (e.g. ductal carcinoma in situ, lobular carcinoma in situ) and defined the pathophysiologic molecular mechanisms responsible for invasion and metastasis (Allred et al. 2001). The authors of these studies have created opportunities for more targeted approaches to the prevention and treatment of breast cancer. Furthermore, the simultaneous evolution of available technologies has resulted in the production of a quite impressive array of molecular targets and biological agents for basic and translational research. With the increasing costs associated with the process of drug development, it appears necessary to focus resources on the target/drugs that are more likely to have an impact on breast cancer management. We present here a comprehensive summary of the accumulating knowledge of these molecular targets. In undertaking this challenging task we have tried to present the information in a useful format in order to provide clinical investigators with generating hypothesis tools that can stimulate the development of appropriate translational studies.

Molecular mechanisms or pathways with established functions in the biology of breast cancer, which constitute the fundamental targets for prognostic and therapeutic approaches, are described in detail. On the basis of their proposed functions, they can be classified into two essential groups: (1) hormonal targets, which are mostly responsible for regulating cell differentiation and epithelial integrity, and (2) non-hormonal targets, which control essential processes responsible for cell growth and movement, cell–cell communications, and the complex interactions between cancer cells and the tumor microenvironment (Fig. 1). This latter group comprises various categories, including (a) signal transduction modulators, (b) cell-cycle regulators, and (c) angiogenesis modulators.

Under the terms ‘hormonal targets’, the prototype being represented by the estrogen receptor (ER), it is reasonable to include the other members of the nuclear receptor family (NRF) whose structures and functions have been clarified (Table 1).

Hormonal targets
NRF in breast cancer: potential targets for prevention and treatment
The past decade has seen a remarkable increase in our knowledge of the structures and functions of steroid hormone receptors through the isolation and sequence analysis of each receptor’s DNA (Mangelsdorf et al. 1995, Nicholson et al. 1995, Hager et al. 2000). It is now established that the receptor proteins from each of the five classes of steroid hormone receptors, notably ER, progesterone receptor (PR), androgen receptor, glucocorticoid receptor (GR), and mineralocorticoid receptor, as well as the non-steroidal hormone receptors for thyroid hormone (TR), 1,25-dihydroxyvitamin D3 (VDR), retinoids (α, β, β), and the so-called orphan receptors, including peroxisome proliferator-activated receptor (PPAR), farnesoid X-activated receptor, and constitutive active receptor, show marked conservation of structure and contain various
functional domains, including those for ligand and DNA binding (Nicholson et al. 1995). The DNA targets of nuclear receptors, known as hormone response elements (HRE), are the sequences through which receptors mediate the control of ligand-responsive genes. Essentially, the receptors ultimately act as ligand-inducible nuclear transcription factors, with interactions between activated receptors and HRE on the DNA modifying gene expression directly (Ma et al. 1999).

The nuclear receptor DNA-binding domain is one of the most prevalent DNA-interacting regions known (Danelian et al. 1992). It is composed of a highly conserved 66 amino acid core domain located centrally in each nuclear receptor, together with a short, non-conserved extension into the hinge region of the receptor. Although some differences exist with respect to the organization and initial cellular localization on the individual classes of hormone receptors, they all ultimately act to stimulate or repress mRNA production from sensitive genes leading to changes in protein synthesis and, finally, cellular function.

The essential roles of ER and PR in the regulation of breast cancer growth have been clearly established (Fisher et al. 1986). These efforts have translated to the design of therapeutic strategies directed at the modulation of these receptors as well as their validation as prognostic and predictive markers in breast cancer. The roles of other members of the NRF in breast carcinogenesis are not clearly defined, although there are indications, at least for some members of the family, of altered expression or activity in breast cancer cells. Herein we review the evidence that supports roles for members of the NRF as therapeutic targets in breast cancer.

Vitamin D receptor

Vitamin D (1α,25-dihydroxycholecalciferol or 1α,25-(OH)₂D₃) plays a significant role in the regulation of cell growth and differentiation. Most of the biologic actions of 1α,25-(OH)₂D₃ are thought to be mediated by the VDR (Haussler et al. 1998).

The human VDR was cloned and sequenced in 1988 by Baker et al. and was subsequently recognized as a member of the superfamily of nuclear receptors that regulate gene expression in a ligand-dependent manner. The VDR shows areas of close homology with the DNA-binding and hormone-binding regions of other steroid hormone receptors. The critical role of VDR in the biology of vitamin D action has been clearly defined at the molecular level (Haussler et al. 1998). The mitochondrial enzyme 25-hydroxyvitamin D(3)-1α-hydroxylase (1α-hydroxylase) plays an important role in calcium homeostasis by catalyzing synthesis of the active form of vitamin D. Following renal production as the hormonal metabolite of vitamin D₃, 1α,25-(OH)₂D₃ functions as the ligand for VDR, with the hormone–receptor complex inducing hypercalcemic and phosphatemic effects that result in normal bone mineralization. VDR not only mediates the action of 1α,25-(OH)₂D₃ in calcium/phosphate translocating tissues, primarily the intestine, but also controls many
Table 1  Nuclear receptor family in breast cancer.

<table>
<thead>
<tr>
<th>Receptor category</th>
<th>Natural ligand</th>
<th>Synthetic ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steroid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td>E</td>
<td>TAM, SERMs</td>
</tr>
<tr>
<td>PR</td>
<td>P</td>
<td>MPA, RU486</td>
</tr>
<tr>
<td>AR</td>
<td>DHT</td>
<td>Hydroxyflutamide, bicalutamide</td>
</tr>
<tr>
<td>GR</td>
<td>C</td>
<td>CP-394531, CP-409069</td>
</tr>
<tr>
<td>Non-steroid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TR</td>
<td>T3</td>
<td>None</td>
</tr>
<tr>
<td>VDR</td>
<td>1α,25-(OH)2D3</td>
<td>1α-(OH)D3, EB1089, CB966</td>
</tr>
<tr>
<td>RAR</td>
<td>RA</td>
<td>ATRA</td>
</tr>
<tr>
<td>RXR</td>
<td>RA</td>
<td>ATRA, 9-cis RA, 13-cis RA</td>
</tr>
<tr>
<td>PPAR</td>
<td>PGJ2</td>
<td>TGZ, LY293111</td>
</tr>
<tr>
<td>FXR</td>
<td>BA</td>
<td>SR-45023A (apomine)</td>
</tr>
<tr>
<td>CAR</td>
<td>PB, E</td>
<td>None</td>
</tr>
</tbody>
</table>

E, estradiol; TAM, tamoxifen; SERMs, selective estrogen receptor modulators; PR, progesterone receptor; P, progesterone; MPA, medroxyprogesterone acetate; AR, androgen receptor; DHT, dihydrotestosterone; GR, glucocorticoid receptor; C, cortisol; TR, thyroid receptor; T3, tri-iodothyronine; VDR, vitamin D receptor; RAR, retinoid nuclear receptor; RA, retinoic acid; 1α-(OH)D3, 1α-hydroxyvitamin D3; ATRA, all-trans retinoic acid; RXR, retinoid X receptor; PPAR, peroxisome proliferator-activated receptor; PGJ2, 15-deoxy-Δ12,14-prostaglandin J2; TGZ, troglitazone; FXR, farnesoid X-activated receptor; BA, bile acid; CAR, constitutive active receptor; PB, phenobarbital.

bioactivities in other major cell systems in the organism, including the immune, neural, epithelial, and endocrine systems (Ozono et al. 1991). In fact, 1α,25-(OH)2D3 appears to dramatically affect the maturation and functions of certain normal and neoplastic epithelial cells (Brenner et al. 1995). Also, the proliferation of a number of epithelium-derived cancer cells (e.g. mammary, prostate, and colon) is inhibited in vitro by 1α,25-(OH)2D3, with some cells being directed toward a more differentiated phenotype (Stoica et al. 1999).

The enzyme 1α-hydroxylase is expressed in multiple extrarenal tissues, including benign breast tissue and breast carcinomas (Friedrich et al. 2001). VDR mRNA has been detected in breast cancer cell lines (Buras et al. 1994, Stoica et al. 1999). The level of expression is higher in well-differentiated, ER-positive cell lines (e.g. MCF-7, T47D) and is lower in poorly differentiated cells (e.g. MDA-MB-231) (Buras et al. 1994). This VDR expression predicts inhibition of cell growth associated with 1α,25-(OH)2D3 treatment and has been described in about 80–90% of breast cancer specimens (Colston et al. 1989, Friedrich et al. 1998). VDR expression may have important prognostic implications (Eisman et al. 1986, Berger et al. 1991).

A retrospective evaluation of ER, PR, and VDR receptor status in 136 patients with primary breast cancer (Colston et al. 1989) showed a positive immunohistochemical staining in 123 (90%) tumors, while no immunoreactivity was detected in 13 additional tumors (10%). Patients with VDR-positive tumors had significantly longer disease-free survival than those with VDR-negative tumors. The presence of VDR expression was not related to patient age, menopausal status, or disease stage, and did not confer a significantly different overall survival rate. In a subsequent comparative study evaluating 34 breast specimens (25 invasive breast cancers, and nine benign breast tissues), Friedrich et al. (1998) reported that the level of VDR expression, measured as the immunoreactive score of VDR intensity, was higher in invasive breast carcinomas than in normal breast tissue (7.28 vs 1.55).

These observations have prompted investigations on the possible role of 1α,25-(OH)2D3 and several of its derivatives in the treatment of breast cancer. Those agents have been found to promote differentiation and inhibit growth in breast cancer cell lines, in particular those expressing VDR (Colston et al. 1989, Fioravanti et al. 1998, Lazzaro et al. 2000, Mehta et al. 2000). The inhibitory effects are associated with down-regulation of cell invasion-associated serine proteases (e.g. urokinase plasminogen activator) and metalloproteinases (e.g. MMP-9) (Koli & Keski-Oja 2000). Furthermore, 1α,25-(OH)2D3 is associated with induction of transforming growth factor β signaling (Yang et al. 2001). The findings of the majority of the studies suggest that the effects of 1α,25-(OH)2D3 are limited to tumors expressing ER (James et al. 1994); 1α,25-(OH)2D3 is known to interact with the estrogen response element at the molecular level and to directly influence ER level (Stoica et al. 1999). In vitro data suggest a potentiation of antiproliferative effects when 1α,25-(OH)2D3 is combined with anti-estrogens, e.g. tamoxifen (TAM) and fulvestran (Wijngaarden et al. 1994, Love-Schimenti et al. 1996).

VDR has been shown to interact with the retinoid X receptor (RXR), forming VDR/RXR heterodimers. Combinations of 9-cis retinoic acid and 1α,25-(OH)2D3 demonstrated an additive antiproliferative effect on retinoid-sensitive cell lines (MCF-7) (Koga & Sutherland 1991, Mackay & Colston 1995, Dawson et al. 1998). Taken together, these data suggest that 1α,25-(OH)2D3 and analogues could be used alone or combined with selective ER modulators or RXR modulators for the treatment and prevention of breast cancer. Interestingly, more recent data suggest that, besides promoting differentiation in in vitro and in vivo models of breast cancer, 1α,25-(OH)2D3 appears to increase chemotherapy-induced cell death when used in combination with doxorubicin or paclitaxel (Wang et al. 2000). New vitamin D analogues (e.g. 1α-hydroxyvitamin D5) may manifest the same effect on
growth inhibition and differentiation regardless of ER status (Mehta et al. 2000).

**Retinoid receptors**

Retinoids nuclear receptors (RARs) are classified as α, β, γ, and RXRs. The actions of all members of this family are modulated by cellular retinol-binding proteins (Mangelsdorf et al. 1994). The human RARβ gene is expressed in three isoforms: β1, β2, and β4. The biologically active RARβ2 isoform is under the regulation of the P2 promoter containing a high-affinity retinoic acid (RA)-responsive element, which is associated with transcriptional activation of RARβ2 by RA in a variety of cells. Xu et al. (1997) analyzed the expression of mRNAs for the three RARs and RXRs in histological sections of specimens from 70 breast cancer patients that included adjacent normal tissue, ductal carcinoma in situ (DCIS), and invasive cancer, by using in situ hybridization. Significantly, a decrease in the number of tissue specimens expressing RARβ was observed comparing DCIS (83.1%) with invasive carcinoma (51.6%). Among the invasive carcinomas, the lower expression was observed in the poorly differentiated tumors (35.7 vs 77.4% in well-differentiated cases). No relationship was found between expression of ER and RARβ (Xu et al. 1997). These findings suggest that the specific loss of RARβ may be an important event during breast carcinogenesis (Ariga et al. 2000, Widschwendter et al. 2000). This theory is further supported by the observation that introduction of an RARβ gene expression vector into RA-insensitive breast cancer cell lines restores RA responsiveness, leading to growth inhibition and induction of apoptosis (Shang et al. 1999).

Preclinical data have demonstrated that carcinogen-induced mammary carcinomas are sensitive to the antiproliferative effects of retinoids (Toma et al. 1997, Bishoff et al. 1998, Wu et al. 2002). ER-positive cell lines have exhibited enhanced sensitivity to retinoids, whereas ER-negative cell lines have, except in a few cases, shown minimal sensitivity (Segars et al. 1993, Klinge et al. 1997). The mechanisms of the antiproliferative effect are still being investigated. In at least some cell lines, apoptosis instead of differentiation seems to be the prevalent mechanism of growth inhibition (Toma et al. 1997).

Selective ligands for RAR and RXR have been tested in breast cancer (Sutton et al. 1997, Budd et al. 1998). All-trans RA has been investigated in small studies of patients with metastatic breast cancer, either alone or in combination with TAM but has demonstrated limited clinical benefit. Ferretinimide, a vitamin A analogue, has been tested in a prospective randomized prevention trial involving women with resected stage I breast cancer (Veronesi et al. 1999). A total of 2972 women were randomly assigned to receive ferretinimide (200 mg/day for 5 years) or no treatment. The trial failed to demonstrate any significant reduction in the incidence of contralateral or ipsilateral breast cancer in the overall population while a trend in favor of the treatment arm was detected in the subset of premenopausal women (contralateral breast cancer, adjusted hazard ratio = 0.66, and 95% confidence interval = 0.41–1.07). These interesting observations suggest the need for additional investigations in high-risk premenopausal women.

Bexarotene is an RXR selective retinoid that has been tested recently in a randomized phase II study (Esteva et al. 2002). Each of the 146 patients was assigned to one of three groups on the basis of her previous treatment for metastatic disease, and treated at two different dose levels (200 vs 500 mg/m² per day). Two groups comprised patients in whom TAM had failed. These two groups were assigned to receive treatment with bexarotene, either alone (TAM refractory) or with continued TAM (TAM resistant). The third group included patients with chemotherapy-refractory disease irrespective of hormone receptor status. Patients assigned to the first two groups achieved the best outcomes with a clinical benefit in 24% (TAM refractory) and 22% (TAM resistant) for the lower dose group. A decrease in serum thyroid-stimulating hormone was reported in 58 patients (40%). Eight (6%) of the patients had clinical hypothyroidism. These data are indicative of a potential therapeutic clinical benefit of such ligands in estrogen-dependent breast cancer and suggest a cross-talk with other members of the NRF, in particular TR.

In further support of this observation, results of recent investigations suggest that nuclear GR/TR/retinoid receptors communicate with each other and with c-erbB membrane receptor tyrosine kinases in the control of mammary epithelial cell proliferation and differentiation, suggesting that efficient growth control requires the co-ordinate interplay of both receptor systems (Natali et al. 1992, Ridley 2001). It would be interesting if future therapeutic approaches with these agents would target more ER-sensitive disease and eventually be combined with modulators of the other members of the NRF.

**Thyroid receptor**

Since Beatson (1896) described the use of thyroid extracts to treat patients with metastatic breast cancer a century ago many studies have investigated the influence of thyroid hormones (tri-iodothyronine (T3), and the prohormone thyroxine) on this form of neoplasia. T3 is able to sustain serum-free proliferation of several cell lines, including breast cancer cells (Zhou-Li et al. 1992, Luong et al. 2002, Dinda et al. 2002). In rodents, mammary gland development (Vonderhaar & Greco 1979) and physiology (Houdebine et al. 1998) are sensitive to T3. In humans, several studies have analyzed the relationship between thyroid status and various diseases, including breast cancer; while no definitive conclusions can be drawn, women with thyroid carcinoma have
an increased incidence of breast cancer (Vassilopoulos-Sellin et al. 1999). This association could be explained either by development of a hypothyroid state associated with dysregulation of the TR function in breast tissue or by a promotion effect of thyroid replacement therapy. To explore the first hypothesis, it is necessary to understand the complex biology of TR, the natural ligand of the thyroid hormone T3. Normal mammary epithelial cells express significant amounts of T3 receptors (TR) (Bhattacharya & Vonderhaar 1977, Selliti et al. 1983), and breast cancer cells contain similar levels of T3-binding activity (Smallridge & Latham 1980, Cerbon et al. 1981). Two TR genes, TRα and TRβ, located on chromosomes 3 and 17 respectively, encode at least four distinct functional receptors: TR1, TR1, TR2, and TR3 (Dressel & Baniahma 2001). TRs are ligand-modulated transcription factors that regulate expression of target genes upon binding to specific nucleotide sequences named thyroid response elements (TREs). TRα and TRβ1 RNA expression is altered in breast cancer (Silva et al. 2002).

The v-erbA oncogene, also known as the avian erythroblastosis virus gene, causes leukemias and sarcomas in chicken. The cellular homologue of the v-erbA oncoprotein is TRα (or c-erbAα) localized on chromosomes bands 3p21–p25, a region in which loss of heterozygosity in breast cancer, among others, has been described (Sap et al. 1986, Dobrovic et al. 1988, Ali et al. 1989, Markowitz et al. 1989). TR and v-erbA are found in the nucleus and bound to naked and/or nucleosomal DNA. TR has the dual role of silencing gene expression in the absence of the hormone and activating genes in the presence of the ligand. Co-amplification of TRα/erbA and c-erbB2/neu oncogenes has been reported in human mammary tumors (van de Vijver et al. 1987). Moreover, several studies have correlated the TRα/erbA expression with certain tumor suppressor genes, such as p53, retinoblastoma, mdm2, or nm23 (Yap et al. 1996, Chang et al. 1997, Barera-Hernandez et al. 1998, Qi et al. 1999).

Until recently, little was known about how T3 acts on mammary epithelial cells at the cellular or molecular level. Gonzalez-Sancho et al. (2002) have studied the putative effects of T3, and its receptor TR1 and, for comparison, the effects of v-erbA on the proliferative capacity of EpH4 cells, a clonal line of immortalized, non-tumorigenic mouse mammary epithelial cells. They reported that TR1 modulated proliferation of EpH4 cells in a T3-dependent fashion and regulated expression of proliferation-associated genes, such as cyclin D1 and T1. In particular, T3 reduced the proliferation of this cell line by inhibiting the expression of cyclin D1 and T1.

TRs and other nuclear hormone receptors can modulate transcriptional activities of each other. This cross-talk can occur via several mechanisms, including formation of heterodimers and competition for cofactors. Several groups have shown that TRs heterodimerize with RXRs, members of the NRF (Yu et al. 1991, Kliwer et al. 1992, Marks et al. 1992, Zhang et al. 1992). TR/RXR heterodimer formation increases the repertoire of target genes that can be regulated by T3, as heterodimers bind to TREs with variable sequences and orientations of half sites (Glass 1994). Notably, addition of both 9-cis-RA and T3 synergistically activated transcription on two different TRE-containing reporters (Rosen et al. 1992). In other cases, however, TR blocked 9-cis-binding to RXR and thereby abrogated retinoid stimulation of target genes (Claret et al. 1996, Forman et al. 1995). Finally, TRs can heterodimerize with other members of the NRF including RAR, PPAR, ER, and VDR (Lucas et al. 1991, Yen et al. 1992, Glass 1994, Schraeder et al. 1994).

**Peroxisome proliferator-activated receptor**

PPARs are transcription factors belonging to the NRF (Mangelsdorf et al. 1995). The three members of the PPAR family of nuclear receptors are α, δ, and γ (Tontonoz et al. 1994). Regulation of gene expression is exerted by the receptors upon heterodimerization with the 9-cis RAR and binding to specific response elements termed peroxisome proliferator-response elements (PPREs). Most PPREs identified to date reside in genes involved in lipid metabolism (Schoonjans et al. 1997). Furthermore, PPARγ is abundant in adipose tissue, where it triggers adipocyte differentiation and lipid storage by regulating the expression of genes critical to adipogenesis (Tontonoz et al. 1994). Although PPARγ is expressed primarily in adipose tissue, it is also expressed in many other tissues and cell types, including colon and breast cancer cell lines and primary and metastatic breast cancers (Mueller et al. 1998, Sarraf et al. 1998, Nwankwo & Robbins 2001).

Ligand activation of PPARγ in cultured breast cancer cells causes morphologic and gene expression changes associated with a more differentiated state (Mueller et al. 1998). There are several known ligands for PPARγ, including the natural prostaglandin 15-deoxy-Δ12,14-prostaglandin J2 (PGJ2), the synthetic anti-diabetic thiazolidenediones, troglitazone (TZG), and the synthetic compound LY293111 (Lehmann et al. 1995, Schwartz et al. 2002). PGJ2 and TZG have the ability to inhibit breast cancer cell growth through modulation of cell-cycle regulators (G1 regulators), including Rb and cyclin D1 (Pignatelli et al. 2001). Some of the growth-inhibitory effects may be related to the reduced aromatase activity in breast adipose tissue. Furthermore, PGJ2 is able to inhibit the erbB-2 and erbB-3 tyrosine phosphorylation. These preliminary data make PPARγ an interesting target for the prevention and treatment of breast cancer.

**Non-hormonal targets**

**Signal transduction process**

The process of signal transduction is often used to indicate the biochemical components that regulate interaction among
which HER2 is recruited as a preferred partner (Ross et al. 1995). Binding of ligands to EGFR, HER3, and HER4 results in formation of homodimeric and heterodimeric receptor complexes, into which HER2 is recruited as a preferred partner (Ross & Fletcher 1999). Members of the human EGFR family, especially HER1 and HER2, are frequently implicated in the pathogenesis of a variety of cancers, including breast cancer (Bacus et al. 1994, Gullick & Srinivasan 1998), HER1 and HER2 activation is associated with neoangiogenesis (Viloria Petit et al. 1997). Both HER1 and HER2 are being actively investigated as potential targets for cancer therapy. One strategy has involved the use of monoclonal antibodies (mAbs) that inhibit receptor function by targeting the receptor’s ectodomain (Cobleigh et al. 1999). Another approach is to use small molecules that possess inhibitory tyrosine kinase activity against one or more members of the HER family (Arteaga et al. 2002). Both classes of agents have been studied in various combinations in clinical trials over the last several years, and updated results have been reported extensively.

A more interesting area of recent investigation is the combination of agents targeting both HER1 (e.g. ZD1839) and HER2 (e.g. trastuzumab); this is based on the in vitro data showing that modulation of HER1 results in inhibition of cell lines with activated HER2 (Arteaga et al. 2002). Moreover, combinations of these agents with angiogenesis modulators, in particular agents targeting vascular endothelial growth factor (VEGF), have been suggested and trials initiated (Pegram et al. 2002). These combinations are extremely attractive because they suggest the possibility of controlling tumor growth and progression more effectively by using agents that target different signal pathways.

**C-kit and PDGF receptor**

The proto-oncogene c-kit is the cellular homologue of the onconege v-kit found in the feline sarcoma virus (Yarden et al. 1987). C-kit encodes for kit (CD 117), a 145 to 160 kDa transmembrane receptor tyrosine kinase that is structurally similar to macrophage colony-stimulating factor and PDGF receptor (PDGFR) (Qiu et al. 1988). Stem cell factor (SCF), the receptor ligand, is also known as Kit ligand, steel factor, and mast cell growth factor (Natali et al. 1992). Expression of C-kit, and the interaction of its product with SCF, are important in the maintenance of normal hematopoiesis, melanogenesis, gametogenesis, and other normal physiological processes. C-Kit also plays an important role in the development of gastrointestinal stromal tumors, small-cell lung cancer, acute myelogenous leukemia, melanoma, breast cancer, and other neoplasms (Wong et al. 1989, Matsuda et al. 1993, Lammie et al. 1994, Hirota 1998).

Several investigators using different techniques have reported variable results concerning c-Kit expression in benign and malignant breast epithelia (Natali et al. 1992a, Matsuda et al. 1993, DiPaola et al. 1997). The general consensus appears to be that breast cancer progression is associated with progressive loss of c-Kit expression. For example, Natali et al. (1992b) observed a ‘high and homogeneous’

### Table 2: Non-hormonal targets in breast cancer.

<table>
<thead>
<tr>
<th>Category</th>
<th>Class</th>
<th>Biomarkers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signal transduction</td>
<td>Growth factors and receptors</td>
<td>EGFR, HER2/neu, VEGFR, PDGFR, c-kit Ras family</td>
</tr>
<tr>
<td>modulators</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell-cycle regulation</td>
<td>Cyclins, CDKs, PS3</td>
<td></td>
</tr>
<tr>
<td>Angiogenesis modulation</td>
<td>VEGF, bFGF</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Proteases, integrins</td>
<td></td>
</tr>
<tr>
<td></td>
<td>COX-2 inhibitors</td>
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</table>

Various intracellular (e.g. membrane, cytoplasm) and extracellular compartments of the cell. These components act as messengers that work in a co-ordinated sequence to regulate cell cycle, cell–cell interactions, movement, and interactions with the surrounding microenvironment (Table 2). The essential components of the signal transduction are represented by (a) growth factors (e.g. epidermal growth factor (EGF) family, platelet-derived growth factor (PDGF), cell-surface receptors, mostly receptor tyrosine kinases, and (c) intracellular signaling pathways (e.g. mitogen-activated protein kinase (MAPK), mitogen-activated protein kinase kinase (MEKK), phosphatidylinositol-3-OH kinase (PI-3K/AKT)). Various components of the process have been investigated extensively and constitute the most utilized model for targeted drug discovery and development.

**EGF family and receptors**

Growth factors and their receptors play pivotal roles in the regulation of epithelial cell growth and differentiation (Aaronson et al. 1991). The HER/erbB family of receptors includes the EGF receptor (EGFR, HER1, erbB-1), the orphan HER2/neu (erbB-2), and the neuregulin/heregulin receptors HER3 (erbB-3) and HER4 (erbB-4). The ectodomain of EGFR, HER3, and HER4 interacts with a specific set of soluble ligands, whereas no ligand has been identified for the HER2 receptor (Salomon et al. 1995). Binding of ligands to EGFR, HER3, and HER4 results in formation of homodimeric and heterodimeric receptor complexes, into which HER2 is recruited as a preferred partner (Ross et al. 1995).
degree of c-Kit expression in 100% (6/6) of normal breast specimens, a 'low and heterogeneous' degree of expression in the epithelium of 78% (28/36) of benign tumors including fibrocytic disease, fibroadenoma, ductal papillomatosis, and gynecomastia, and in the epithelium of 13% (10/80) of primary breast cancer specimens, and 3% (1/40) of metastatic breast cancer specimens. DiPaola et al. (1997) demonstrated the presence of c-Kit mRNA in breast cancer cells by reverse-transcription PCR and Western blot analysis.

The PDGF family is composed of four members, PDGF-A, -B, -C, and -D, which signal through the α and β tyrosine kinases. PDGFs are composed of two chains held together by disulfide bonds and present in dimeric forms AA, AB, BB, CC, and DD (Hammacher et al. 1988, Westermark 1990). PDGF is a ubiquitous growth factor driving cell proliferation during normal development and in a variety of pathological conditions. The PDGF-dependent mitogenic pathway has been implicated in pathological conditions such as cancer and connective tissue disorders. Interest in the role of PDGF in cancer stems from the finding that the transforming protein of the simian sarcoma retrovirus (v-sis oncogene product) is highly related to the β-chain of PDGF (c-sis proto-oncogene product) (Westermark 1990). Expression of the v-sis or c-sis genes in cells expressing the PDGFR has been shown to cause cellular transformation and tumorigenicity in vivo. Dysregulation of PDGF signaling in tumors can lead to either autocrine or paracrine stimulation of cell growth. The PDGFR pathway has been implicated in the growth of tumors of mesenchymal origin, most notably sarcomas and gliomas (Lokker et al. 2002). The PDGFRβ has a ligand-activated protein kinase, PDGF BB, which is secreted in most human breast carcinoma cells; PDGFRβ has been demonstrated in malignant breast tissue and mostly localized in the periepithelial stroma (Coltrera et al. 1995, Bhardwaj et al. 1996). PDGF concentrations in plasma and tissue of patients with breast cancer have indicated that high PDGF levels predict for shorter survival times and a lower response to chemotherapy (Ariad et al. 1991).

STI571 (imatinib mesylate), a phenylaminopyrimidine derivative, is a member of a new class of drugs collectively known as signal transduction inhibitors (Buchdunger et al. 2000, Heinrich et al. 2000). More specifically, it is an inhibitor of several tyrosine kinases that are believed to play a role in the proliferation of tumor cells (Ricotti et al. 1998, Tian et al. 1999). These include the tyrosine kinases associated with bcr-Abl, PDGF and c-Kit. STI571 is presently being tested in metastatic breast cancer associated with expression of either c-Kit and/or PDGFR. Interestingly, preliminary investigations into the signal transduction pathway showed that SCF and EGF (natural ligands for c-kit and HER1) activated both the RAS-MAPK kinase and phosphatidyl-inositol-3-kinase (PI3 kinase) pathways (Heuchel et al. 1999). These results suggest that coexpression of SCF and c-Kit may be involved in the response to erbB ligands through activation of MAPK and PI3 kinase (Hines et al. 1999). Subsequently, Peng et al. (2002) demonstrated that the combination of STI571 with mAbs targeting the HER-B family (referred to as trastuzumab and cetuximab) results in additive growth inhibition of STI571-sensitive breast cancer cell lines (e.g. SKBR3 and MCF-7). Moreover, the triple treatment combination resulted in greater growth inhibition (80% in SKBR3 and 60% in MCF-7). In spite of these provocative data, the role of modulation of c-Kit and PDGFR with STI571 or more selective modulators is still to be established (Oastman & Hedln 2001).

Ras proteins

Ras is a membrane-bound GTP/GDP-binding (G) protein which serves as a 'molecular switch' converting signals from the cell membrane to the nucleus (Valencia et al. 1999b). These chemical signals lead to protein synthesis and regulation of cell survival, proliferation, and differentiation. The Ras family includes several distinct members, such as Ras (H, K, M, N, and R), Rap (1 and 2), and Raf, which share approximately 50% sequence identity (Boguski & McCormick 1993). This family shares at least 30% sequence identity with several other small monomeric G protein families, such as the Rho/Rac/CDC42, Rab/Ypt, Ran, Arf, and Rad (Hill et al. 1995, Wittinghofer & Herrmann 1995). Ras is quiescent in the GDP-bound form, but upon activation by external ligands it transforms to the active GTP-bound form (Buday & Downward 1993). Membrane interactions require prenylation, the covalent addition of either farnesyl (15 carbon) or geranylgeranyl (20 carbon) groups to conserved carboxy-terminal cysteine residues of certain proteins (CAAX sequence) (Goldstein & Brown 1990). Prenylation is catalyzed by three enzymes: farnesyltransferase, geranylgeranyltransferase type 1, and geranylgeranyltransferase type 2. Activated Ras utilizes several downstream effectors, of which the best characterized are Raf-1, Rac, Rho, and PI3 kinase.

Rho-GTPases have been investigated extensively because of their critical roles in several cellular processes, such as cell morphology, and regulation of gene expression, cell proliferation, and survival (Qiu et al. 1995, Van Aelst & D'Souza-Schorey 1997, Hall 1998). Rho proteins are important in maintenance of normal epithelial polarity, regulation of morphology and motility, and formation of cell junctions (Clark et al. 2000, Ridley et al. 2000, Keely 2001). Rho family proteins were initially cloned on the basis of their similarity to the ras oncogene, and a total of 18 members have so far been identified. There are no reports of mutated, constitutively active forms of Rho proteins in tumors, as there are for Ras, but several members of the family are overexpressed in human tumors (Schnelzer 2000). RhoC and RAC1 have been shown to be overexpressed in breast cancer (van Golen et al. 2000, Schnelzer 2000, Kleer et al. 2002).
Rad, another member of the family, has been demonstrated to act as an oncogenic protein through interaction with nm23 (Tseng et al. 2001). van Golen et al. (2000) have demonstrated that RhoC GTPase was overexpressed in 90% of inflammatory breast cancer (IBC) and in only 38% of non-IBC (P = 0.0095). RhoC-GTPase transfaction in immortalized breast cells was associated with transformation and increased levels of VEGF, basic fibroblast growth factor (FGF) (bFGF), interleukin-6 (IL-6) and IL-8 in conditioned medium suggesting modulation of angiogenic factors. Significantly, RhoC expression may be a predictor of recurrence for patients with axillary node-negative breast cancer, even in lesions less than 1 cm in size (Kleer et al. 2002). These data suggest that overexpression of RhoC may function as an oncogene in the early stage as well as in advanced disease, indicating that these proteins may be involved in various stages of carcinogenesis. Some studies have already provided insight into the effect of targeting Rho protein function in various models. In particular, farnesyl transferase inhibitors, by preventing lipid modification of Rho proteins, have been demonstrated to be capable of reversing the malignant phenotype associated with overexpression (van Golen et al. 2000). These agents have already demonstrated clinical activity in advanced breast cancer and have been included in proposed combination regimens for neoadjuvant treatment of locally advanced breast cancer (LABC) and IBC.

Cell-cycle and apoptosis modulation

Control of cell proliferation and activation of apoptosis regulation are tightly regulated key mechanisms of normal growth and development. These processes are essential also for cancer cell growth and progression. Cyclins and cyclin-dependent kinases (CDKs) have demonstrated importance in cell-cycle regulation at the G2/M checkpoint. Exposure of cells to stress activates the p53 tumor suppressor protein. This activation is cardinal for the efficient response of cells to stress stimuli, such as DNA damage and hypoxia (Giacca & Kastan 1998). The response takes the form of growth arrest, senescence, or apoptosis (Askar et al. 1999). Loss of p53 function, either by mutations or by deregulation of regulatory proteins, is often detrimental, leading eventually to development of cancer (Levine 1997). The most common mutations are represented by missense mutations that result in conformational alteration and dysfunction of p53 protein (Ko & Prives 1996).

The key inhibitor of p53 is the mdm2 proto-oncogene. mdm2 (hdm2 in humans) inhibits the transcriptional activity and growth suppression ability of p53 through an autoregulatory feedback loop (Blagoslklynny 2000). Importantly, mdm2 promotes p53 for degradation through the ubiquitin system (An et al. 1998) by acting as an E3-ubiquitin ligase. The nuclear export of p53 is believed to be important for mdm2-dependent degradation (Roth et al. 1998), although degradation within the nucleus has also been observed (Yu et al. 2000, Xirodimas et al. 2001). Nucleo-cytoplasmic shuttling depends on one or more nuclear export signals of p53 (Zhang & Xiong 1999) and requires the ubiquitylation of p53 by mdm2 (Boyd et al. 2000).

p53 mutations have been documented with higher frequency in LABC, including IBC, than in early breast cancer (38 vs 18%), suggesting that this may be a late phenomenon in breast cancer tumorigenesis. The presence of p53 dysfunction correlates with more aggressive tumors, early metastasis, and decreased survival rates (Pharoah et al. 1999).

Some studies have suggested that loss of p53 function is related to worse prognosis and lower chemosensitivity in breast cancer (Elledge et al. 1993, Bergh et al. 1995, Aas et al. 1995, 1996, Elledge & Alld 1998). One series, which used a molecular approach of constant denaturant gel electrophoresis followed by direct sequencing of PCR, showed that "de novo" resistance to doxorubicin in LABC was associated with specific p53 mutations (Aas et al. 1996).

p53 function can be restored by restoring the confor-
Angiogenesis modulation

In order for a tumor to grow beyond a certain size (2 mm³), it must develop a network of blood vessels to supply nutrients and oxygen and to remove waste products. Angiogenesis is complex in both physiological and pathophysiological processes, and is regulated through production of several pro-angiogenic and anti-angiogenic factors (Carmeliet 2000). Inhibitory factors, such as endostatin, normally predominate, but various signals can activate the ‘angiogenic switch’ (Skobe et al. 1997, Westphal et al. 2000). Angiogenic factors, in particular bFGF and VEGF, activate endothelial cells, which leads to secretion and activation of matrix metalloproteinasises (MMPs) and plasminogen activators (e.g. urokinase plasmin activator complex (uPA-PAI)) (Yancopoulos et al. 2000).

This results in degradation of the basement membrane, which allows the endothelial cells to invade the surrounding matrix. Integrin molecules are essential for the interaction with the extracellular matrix and basement membrane and in regulating several signaling and intracellular processes that control cell growth (e.g. CDKs). Other transmembrane proteins appear to be involved in the complex interaction with the extracellular matrix, including CD44, a hyaluronate receptor (Bourguignon 2001, Okamoto et al. 2002). Several membrane glycoproteins distributed in the basement membrane, know as lamins (e.g. laminin-5), interact with integrins and participate in the regulation of cell function (e.g. invasion). Subsequently, endothelial cells migrate, proliferate (stimulated by growth factors such as bFGF and VEGF), and eventually differentiate to form a new, lumen-containing vessel. Finally, the endothelial cells deposit a new basement membrane and secrete growth factors, such as PDGF, which attract supporting cells to stabilize the new vessels (Carmeliet 2000). This complex process involves several steps and factors, whose potential roles as molecular targets in breast cancer have been studied.

VEGF, a highly conserved, soluble dimeric glycoprotein of M, 34 000–45 000 (VEGF165), is a strong endothelial cell-specific mitogen and also a potent vascular permeabilizing agent (Robinson & Stringer 2001). Expression of VEGF increases angiogenesis and tumor growth and reduces experimental metastasis in nude mice. Retrospective clinical data have clearly established that higher levels of cytosolic VEGF represent a strong independent prognostic factor in node-negative as well as node-positive breast cancers (Linderholm et al. 1998). Binding of VEGF to its receptors, known as VEGF receptor 1 (Flt-1) and 2 (KDR/fek-1), begins the signaling cascade that regulates cellular events involved in new blood vessel formation. The major receptor for VEGF is Fek-1, which is among a number of endothelial cell growth factor receptors that are thought to be involved directly or indirectly in angiogenesis (Shawver et al. 1997). Flk-1, like many other growth factor receptors, has an extracellular ligand-binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain. Upon VEGF binding, the receptor dimerizes, the tyrosine kinase is activated, and the receptor becomes autophosphorylated (Ullrich & Schlessinger 1990). The cascade triggered by receptor tyrosine kinase activation modulates cellular events such as proliferation, differentiation, and morphogenesis.

In vitro, neutralizing antibodies against Flk-1 inhibit mitogenesis (Rockwell et al. 1995). Similarly, ribozymes that cleave flk-1 mRNAs reduce the growth of human microvasculature endothelial cells, presumably by decreasing the number of receptors on the cells (Hasan & Jayson 2001). In vivo, Flk-1 receptors that lack the intracellular kinase domain have been shown to block the activation of the endogenous Flk-1 receptor activity in cultured cells, inhibiting the growth of tumors implanted subcutaneously into nude mice (Millauer et al. 1994). Therapeutic strategies targeting VEGF receptor-activated signaling through the use of neutralizing antibodies (rhuMab-VEGF), ribozymes or small molecules with tyrosine kinase inhibitory activity are presently in the advanced phase of testing in metastatic and primary breast cancers.

Proteinases such as MMPs, serine proteinases, and cysteine proteinases are believed to play a critical role in cancer
progression. In particular, the urokinase plasminogen activation system, constituting uPA, its specific receptor uPAR, and its inhibitors, PAI-1 and PAI-2, has in experimental in vivo tumor models been shown to be involved in these processes (Mazar et al. 1999, Fisher et al. 2000, Fox et al. 2001, Frandsen et al. 2001). In situ hybridization and immunohistochemical studies of human breast cancer tissue have shown that uPA mRNA and protein are expressed mainly by myofibroblasts surrounding nests of cancer cells, whereas uPAR is expressed mainly by infiltrating macrophages, revealing the role of stromal cell components of the tumor tissue in the proteolytic activity (Pyke et al. 1993, Nielsen et al. 2001). Overexpression of uPA and PAI-1 requires constitutive p38MAPK (Huang et al. 2000). Furthermore, maintenance of activated p38MAPK is associated with activation of αv integrin signaling, suggesting a functional link with uPA/PAI-1 (Chen et al. 2001, Degryse et al. 2001, van der Pluijm et al. 2001). High levels of uPA in the primary tumor have been found to have prognostic significance including axillary node-negative breast cancer (Knopp et al. 1998, Malmstrom et al. 2001). High levels of PAI-1 have demonstrated prognostic significance in primary breast cancer independently of the lymph nodal status (Harbeck et al. 2001). These markers have been validated in a prospective study in which women were selected for treatment on the basis of the level of expression of uPA/PAI-1 (Janicke et al. 2001). Specific inhibitors of the uPA system have been developed and found to suppress tumor growth in animal models (Sturzebecher et al. 1999, Guo et al. 2000, Probst et al. 2001, Rabbani & Gladen 2002). Some of them are entered in early phase clinical investigations and may represent an interesting approach to early breast cancer.

The integrin family is composed of pairs of α and β transmembrane subunits, which are selected from at least sixteen α and eight β subunits to form more than twenty αβ heterodimeric receptors on the cell surface (Schwartz 1997, Giancotti & Ruoslahti 1999). Laminin-5, an extracellular matrix protein that plays a key role in cell migration and tumor invasion, has been shown to be a ligand for integrins α3β1, α6β4, and α2β1 (Giannelli & Antonaci 2000, Kiosses et al. 2001, Lohi 2001). Maspin, a member of the serpin family with suppressor activity, also interacts with members of the integrin family (Seftor et al. 1998).

Integrins can directly activate many intracellular signaling events after stimulation by extracellular matrix proteins or by antibodies that bind to specific sites of integrins (Lafrenie & Yamada 1996, Morini et al. 2000, Rabinovitz et al. 2001). Integrins and their cytoplasmic tails are involved in forming large complexes of signaling molecules that include, among others, Src family kinases (Kaplan et al. 1994), signal transduction molecules such as growth factor receptors (Plopper et al. 1995), Ras (Kapron-Bras et al. 1993), nerve factor-xB (Yebra et al. 1995), and PI3 (Chen et al. 1994). The integrins α3β1 and α6β4 have been associated with the process of invasion and metastasis in mammary carcinogenesis (Mercurio et al. 2001). Increased expression of α6β4 is of prognostic relevance in breast cancer, in particular when associated with increased levels of laminin-5 (Tagliabue et al. 1998). These data suggest important roles for some members of the integrin family, in particular α6β4 and possibly α3β1, in the process of invasion and metastasis and their potential use as therapeutic targets of specific modulators.

Another intriguing component of the angiogenesis process involves prostaglandins, which appear to be involved in cell proliferation, migration, and ability to form new vessels. Cyclo-oxygenase (COX) inhibitors interfere with the prostaglandin synthesis pathway. Of particular interest have been the COX-2 inhibitors, which affect the COX-2 enzyme in the above-described pathway. In vitro and mouse studies have demonstrated that COX-2 inhibitors slow tumor progression by inhibiting tumor cell migration and angiogenesis (Rozic et al. 2001).

Ristimaki et al. (2002) examined breast tumor specimens by immunohistochemistry from 1576 patients and demonstrated that elevated COX-2 expression is associated with a large tumor size, high histological grade, negative hormone receptor status, high p53 expression, presence of the HER2/neu oncogene, and axillary lymph node metastases. They concluded that COX-2 expression is associated with poor prognostic features and predicts for a poor outcome in primary breast cancer. Based on these data, COX-2 has become one of the most promising targets and clinical trials have been designed testing COX-2 inhibitors (e.g. celecoxib) for the treatment and prevention of breast cancer.

Conclusions
The proliferation of knowledge on the pathophysiology mechanisms of breast cancer has provided exponential growth in the number of biomolecular markers. Furthermore, the process of targeted drug design has become faster and more sophisticated, providing a variety of agents that target these markers for in vivo testing in animal models as well as clinical trials. The excitement among clinicians and scientists regarding the increasing treatment opportunities is tempered by concern that resources are inadequate to bring the majority of these agents to advanced clinical testing. The challenges, then, are to select the most promising agents to be tested and the appropriate clinical setting for such tests. In this review, we have utilized a rationale approach to describing the more widely recognized molecular targets in breast cancer. Drugs that modulate the NRF have not been investigated extensively so far, and such investigations can increase the opportunities for true ‘endocrine’ approaches to the prevention and treatment of breast cancer. Moreover, agents that modulate apoptosis and angiogenesis represent an exciting area of investigation, particularly in carefully selected combination regimens.
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