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Integrating signals between cAMP and MAPK pathways in breast cancer

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1. ABSTRACT

Breast cancer is one of the most common malignancies in Western society. Localized breast cancer, before it spreads, can be cured by surgery. However, the high mortality rate associated with breast cancer is due to a propensity of the tumor to metastasize when the primary tumor is small or undetectable. Although steroid receptor status has been recognized as the most precise predictor of response to hormone therapy, a significant number of tumors expressing these receptors metastasize and patients do not respond to the antihormone therapy. The mechanism leading to breast cancer progression and resistance to the hormone therapy is not completely understood at the present time. Compelling evidence shows that hormone-bound steroid receptors in breast cancer cells activate complex signaling networks, which include MAPK- and G protein-dependent pathways. These responses, which occur within seconds or minutes after steroid administration, are not due to changes in gene expression. Depending on cell systems, steroid activation of these networks leads to different and profound effects on extra nuclear and nuclear events. In such a way steroids foster cell cycle, reduce apoptosis and stimulate cell migration of target cells. All these processes are deregulated in breast cancer. In this review we will discuss new aspects of signaling pathways activated by steroids and their integration with other pathways in breast cancer. Recent findings on the discovery of compounds specifically interfering in such a complex network will be presented.

2. INTRODUCTION

Breast cancer is very common in developed countries, with one in ten women developing the disease and half of those dying of it. The status of steroid receptors is a well-established prognostic marker in breast cancer. Estradiol receptor alpha (ER alpha) has been implicated in the progression of breast cancer, and this is corroborated by the finding that about 60-70% of human breast cancers are ER alpha-positive (1). ER alpha status predicts a favorable disease outcome. Most patients with ER alpha-positive breast cancer receive tamoxifen as adjuvant endocrine therapy (2). Survival of tamoxifen-treated patients is longer for women with cancer with ER alpha amplification than for women with ER alpha expressing cancer without amplification (3). However, although tamoxifen treatment has improved the outcome from breast cancer, many patients become resistant to the hormone therapy and develop metastatic breast tumors. Several mechanisms have been proposed to explain the causes of breast cancer resistance to endocrine therapy. These include expression of steroid receptor variants, ligand-independent activation of steroid receptors, over-expression and activation of tyrosine kinases, most notably ErbB2 (4), and signaling effectors, such as AKT (5).

Steroid hormones control proliferation and survival of breast epithelial cells. This activity has been so far attributed to the interaction of steroids with their cognate

receptors and the consequent regulation of gene transcription (6). In addition to the well-studied nuclear function, ERs, progesterone receptor (PgR) and androgen receptor (AR) participate in extranuclear and membrane-mediated signaling events (7). Such a non genomic action has been linked to rapid responses elicited by steroid hormones and involves activation of Src, mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3-K), protein kinase C (PKC) and etherotrimeric G-proteins in cytoplasm or membrane of target cells (8). Furthermore, the extra-nuclear mechanism regulating the cross talk between ER alpha and EGFR in cytoplasm of breast cancer cells has been recently analyzed (9). Interestingly, important biological responses such as DNA synthesis and cytoskeleton changes leading to cell migration can occur in the absence of transcriptional activity or nuclear localization of steroid receptors (10-12). Depending on the cell type and experimental conditions, steroid action may depend on integration between extranuclear and nuclear receptor activities (13).

In this review, we discuss new concepts of cross talk between steroid receptor and signaling effectors accounting for the non genomic actions of steroids. In particular, we highlight recent developments unraveling the intricate signaling network regulated by steroids in breast cancer and the integration of these pathways in the cell. Elucidating the details of these programs should provide a more rational approach to breast cancer therapy.

3. BREAST CANCER: A GENERAL OVERVIEW

Breast cancer remains a widespread disease. In 2004, there were 371,000 new cases of breast cancer diagnosed and 129,900 breast cancer-related deaths in Europe (14). Nevertheless, a decline in mortality rate has been observed during the last few years (15). This decline is due to mammographic screening, more precise diagnosis, and an increase in the number of women receiving the best treatment for their condition, like the extensive use of tamoxifen (16).

The causes leading to breast cancer and the identification of prevention strategies are still elusive. Association of the risk of breast cancer with age at first birth and parity was proposed several years ago (17) and confirmed by subsequent studies (15). Additional risk factors have been added in recent years. These include genetic factors, geographical location, exposure to ionising radiation, particularly during puberty, absence or short lifetime duration of breastfeeding (typical of women in developed countries), use of oral contraceptives, hormone-replacement therapy, high body-mass index and dietary factors, such as alcohol abuse. Progression from healthy mammary tissue to invasive carcinoma is still a debated process. The pre-neoplastic potential of benign, proliferative lesions of breast and dysplastic changes present in different non-malignant breast diseases is not defined. To date, *in situ* carcinomas (either ductal or lobular) are morphologically identifiable as neoplastic transformation, whereas stromal invasion and metastasis to regional lymph nodes or distant organs are the hallmarks of developed breast cancer.

The best approach to breast cancer therapy remains targeting the disease at the earliest stages of development. Tamoxifen, a selective estrogen receptor modulator (SERM), has been largely used because of the data from laboratory models and its ability to prevent contro-lateral breast cancer (18). Although the role of tamoxifen as a chemopreventive for women with high risk of breast cancer is generally accepted, what degree of risk is appropriate for its use remains unclear. In addition, tamoxifen induces increased risk of endometrial cancer and other side effects because of its partial agonist activity (19). Thus, other molecules such as Raloxifene and aromatase inhibitors have been developed. Raloxifene is also a SERM largely used in the treatment of osteoporosis in postmenopausal women. It reduces the incidence of breast cancer in osteoporosis trials and does not exert estrogen-like activity in uterus of rodents. Unfortunately, like tamoxifen, it increases thromboembolic events. Aromatase inhibitors are more effective than tamoxifen in preventing contro-lateral breast cancer and in the adjuvant treatment of early-stage disease. Aromatase inhibitors, however, do not suppress the levels of estradiol in premenopausal patients (18). It is noteworthy that tamoxifen acts through ER, and that only ER-positive breast cancers were reduced in the tamoxifen prevention trials. Its use is not suitable for women with BRCA1 mutations who develop ER-negative breast cancer or in patients with ER-negative breast cancers overexpressing ErbB2/HER2/neu. Drugs targeting other pathways involved in breast carcinogenesis, such as trastuzumab (Herceptin), an antibody against ErbB2, or oral tyrosine kinase inhibitors are used in therapy and new molecules with more specific action are being investigated in laboratory models of breast cancer (8, 20).

In conclusion, the general trend is now to identify new molecular targets in tumors and their neighboring cells and to increase targeted therapy of breast cancer.

3.1. Steroid receptors in breast cancer

Several years ago, Beatson observed that oophorectomy caused tumor regression in advanced breast cancer (21). This seminal finding opened the way to the study of the role of estradiol in this disease. After 70 years, an ER was identified (22) and purified by affinity chromatography (23). This receptor was detected in breast tumors (24) and is now an established prognostic marker. Its expression determines whether or not tamoxifen should be given as adjuvant endocrine therapy.

A second ER was later identified (25) and named ER beta to distinguish it from the original receptor, ER alpha. Two human ER beta isoforms of 530 and 485 amino acids have been described (26-28). The 530 amino acid form is generally believed to be the mature full-length ER beta (27-29).

Although estradiol is the main steroid implicated in breast cancer progression, much evidence points to progesterone as an important factor in the progression and maintenance of the neoplastic phenotype in the mammary gland (30). In fact, clinical data have demonstrated a higher risk of breast cancer in patients under hormone replacement

therapy using a combination of estrogens and progestins as compared with those using estrogens alone (31, 32). PgR, like ER, represents a target in the therapeutic approach to breast cancer (33). Accordingly, recent data raise the possibility that anti-progesterone treatment may be useful for breast cancer prevention in individuals with BRCA1 mutations, which predispose to breast and ovarian cancers (34).

PgR in rodents and humans exists as two isoforms, PgR-A and PgR-B. The two isoforms are produced from a single gene by translation initiation at two distinct start codons under the control of separate promoters (35). PgR-A is a truncated form of PgR-B. In humans, the N-terminal 164 amino acids of PgR-B are missing in PgR-A. Although the two forms of PgR have similar structures and identical DNA and ligand binding domains, *in vitro* studies using a progesterone-responsive transcription system reconstituted in mammalian cells revealed that PgR-A and PgR-B are functionally different. In most cases, PgR-B acts as a potent activator of transcription of target genes, whereas PgR-A acts as a dominant repressor of transcription regulated by PgR-B as well as other nuclear receptors (36).

Although a multitude of molecules involved in breast cancer biology, particularly ErbB2 and mutated BRCA1, are used as markers, determination of steroid receptor status remains an important prognostic assay. Overexpression of ER alpha is a well-established prognostic and predictive factor in breast cancer patients (2). More importantly, a large subset of breast cancers shows a single-gene amplification of the ER alpha gene, thus suggesting that this amplification may be a common mechanism in proliferative breast disease and a very early genetic alteration in breast cancer progression (3). Expression of PgR serves as a functional assay because it indicates that the ER transcriptional pathway is intact. When biochemical ligand binding assays indicate concentrations of 10 fmol/mg cytosol protein or more, breast cancers are generally considered ER-positive and PgR-positive for clinical purposes. ER and PgR status can also be evaluated using immunohistochemistry (IHC). Unlike chemical assays, IHC does not require destruction of tissue specimens; in addition, it shows ER tissue distribution. For these reasons, it has become the preferred method for determining ER/PgR status in breast cancer specimens. Quantitative methods using computer-aided image analysis are being developed to improve the accuracy of IHC.

4. SIGNALING BY STEROID RECEPTORS

4.1. Classical and rapid response models of steroid action

Steroid hormones influence many processes in mammals, including cell growth, cardiovascular health, bone integrity, immunity, cognition, and behavior. Evidence collected in the last few years indicates that regulation of these effects may be mediated by a complex interface between modulation of signaling cascades and control of gene expression. Receptors in the cell nucleus regulate gene expression, whereas classical receptors localized in close proximity to the cell membrane or in the extranuclear compartment of cells activate signal transduction (7, 10). Transcriptional effects of steroid hormones usually occur via ligand-dependent binding of receptors to target gene promoters as part of a pre-initiation transcription complex, which leads to chromatin remodeling and ultimately regulates gene expression (37). The resulting fluctuations in mRNAs and the proteins they encode take place within hours following hormonal exposure. In contrast, steroid activation of signal transducing pathways occurs within seconds or minutes. These rapid effects are insensitive to RNA and protein synthesis inhibitors. Almost all the members of the steroid hormone family, from the corticosteroids (glucocorticoids and mineralocorticoids) to the sex steroid hormones (estrogens, progestins, and androgens), exhibit rapid, non genomic actions. These range from activation of Src, MAPKs, adenylyl cyclase and PI3-K to rises in intracellular-calcium concentrations (38-45).

Much evidence shows that steroid stimulation of breast cancer cells rapidly induces G-protein activation and generation of a second messenger such as cAMP and cGMP (46). Although controversial findings have been reported about the nature of receptors mediating these responses, G protein activation by steroids leads to stimulation of various signaling effectors (46) and release of growth factors with consequent activation of their cognate receptors (47). Depending on the cell context, these signals are related to different effects of steroid hormones, such as proliferation, survival, migration and differentiation.

4.2. Role of cAMP pathway in breast cancer

Several years ago, Szego & Davis reported a rapid, acute elevation of uterine cAMP by estradiol treatment of rats (48). Subsequent studies indicated that estradiol treatment of human prostate tissue greatly increases the intracellular cAMP (49), and findings in ER-positive MCF-7 breast cancer cells showed that estradiol enhances intracellular cAMP production through adenylyl cyclase activation and stimulates cAMP response element (CRE)-mediated gene expression (50). In agreement with these observations, a role for cAMP/protein kinase A (PKA)-dependent pathway in the estradiol-regulated cyclin D1 transcription of ER-positive ZR-75 breast cancer cells has been proposed (51). Altogether, these studies show that signals resulting from activation of G-protein and cAMP-signaling pathways contribute to gene regulation by estradiol.

In addition to being produced in response to steroids and to regulating CRE-mediated genes, cAMP plays a role in the ligand-independent activation of steroid receptors. In fact, 8-Br cAMP treatment of cells transfected with a chicken PgR expression vector and a PgR-responsive reporter causes hormone-independent, but receptor-dependent activation of the reporter (52). These findings have been explained by the observations that cAMP increases phosphorylation of the steroid receptor coactivator-1 (SRC-1; 53-54). In addition, cAMP is also involved in resistance to steroid antagonists that frequently develops in breast cancer, since it enhances the ability of antiprogestin to activate gene transcription mediated by PgR-B in T47D breast cancer cells (55, 56).

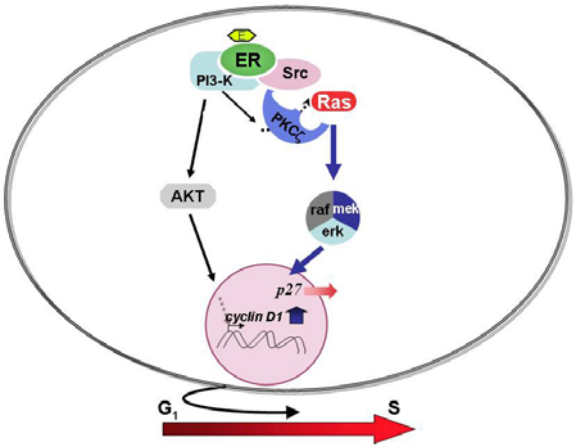

Figure 1. Estradiol activation of signaling effectors is responsible for cell cycle progression in ER-positive breast cancer cells. In breast cancer cells, estradiol rapidly induces the assembly of a complex made up by ER, Src and PI3-K. Through PKC zeta, Ras is also recruited to the complex and the Ras-dependent kinase cascade activated. Stimulation of PI3-K and Ras-dependent cascade leads to increased cyclin D1 transcription and p27 nuclear exclusion. These events are responsible for the G1/S transition of cells.

The role of cAMP in mammary carcinoma cell proliferation has also been investigated. Initial reports indicated that dibutryl-cAMP in conjunction with arginine suppresses the proliferation of MCF-7 cells (57). Subsequently, it was confirmed that elevation of cAMP levels produces substantial effects in MCF-7 cells. Addition of 8Br-cAMP or expression of mutant (Q227L)-activated G alpha$_s$ in MCF-7 cells did indeed block the ability of these cells to grow in an anchorage-independent manner, and stable transfection of activated-G alpha$_s$ in MCF-7 cells reduced the ability of these cells to form tumors in athymic mice (58). These findings indicate that cAMP may be crucial in preventing the expression of transformed phenotype in mammary epithelial cells. In addition, G protein coupled receptor 30 (GPR30) expression correlates with progestin-induced growth inhibition in different breast cancer cells and GPR30 is critical for progestin-induced growth inhibition (59).

It is now largely accepted that estradiol and progestin treatment of breast cancer cells rapidly generates cAMP. This action results from G protein activation and signaling is then transmitted to various effectors, including PKA, PKC, MAPK and PI3-K (46). Although the importance of these signals in the cellular action of sex steroids *in vitro* and *in vivo* is well documented, the nature of receptors mediating these events is still debated. Some models propose the involvement of classical steroid receptors, which initiates signaling cascades by association with the scaffold protein, caveolin-1 (60) and a variety of proximal signaling molecules, including G proteins (61-63), Src (39, 42, 64), PI3-K (11, 43, 65), MNAR (66), PKC zeta (45) and Shc (67). Other candidates in mediating these events are represented by traditional G protein-coupled receptors (GPRs). One of these receptors has recently been identified by different groups as GPR30, an orphan GPR (68, 69).

4.3. MAPK signaling in breast cancer

MAPK circuits transmit and amplify signals involved in a plethora of cell responses. These pathways are indicators of the intensity and length of signals induced by growth factor, steroid hormones, and ligands of G protein coupled receptors. Three major MAPK pathways exist in human tissues, but ERK-1 and -2 are the most relevant to breast cancer, and several studies demonstrate that they are frequently activated in breast cancer (70). A number of investigators have now studied the expression of activated MAPK in human breast cancer tissues by enzymatic assay and immunohistochemical techniques. In one half of breast tumors MAPK is more active than in the surrounding benign tissue. Studies also show a trend toward higher MAPK activity in primary tumors of node-positive than in node-negative patients; this up-regulation is not caused by Ras mutations, but results from enhancement of growth factor pathway activation (70).

Estradiol, progesterone and androgens very rapidly activate MAPK in breast, prostate and colon cancer cells (39, 40, 42, 64, 71, 72). This activation depends on the stimulation of the Src/Ras cascade by sex steroids and has a proliferative role as demonstrated by experiments with chemical inhibitors and signaling effector mutants (10, 12, 42). In breast cancer cells, estradiol triggers direct interaction of classical ER alpha with the SH2 domain of Src, whereas androgens trigger AR interaction with the SH3 domain of Src (42). Estradiol activation of the Src axis occurs alongside PI3-K. Hormone stimulation of MCF-7 cells induces the assembly of a multi-molecular complex made up by ER, Src and p85 alpha, the regulatory subunit of PI3-K, which triggers activation of the Src and PI3-K-dependent pathways. Hormone-activated PI3-K targets Akt and PKC zeta. Once activated, Akt increases cyclin D1 transcription, whereas PKC zeta controls Ras recruitment to the ER/Src/PI3-K complex, Erk-2 nuclear translocation and the consequent release of p27 from cell nuclei. By this interplay between signaling effectors and cell cycle regulators, cells enter the S-phase (43, 44). These conclusions have been highlighted by recent findings showing that specific interference in the sex steroid receptor/Src interaction by new, cell-permeable molecules inhibits the growth of mammary tumor and prostate tumor cells *in vitro* and in nude mice (8, 20).

Figure 1 depicts the estradiol control of cell cycle progression through signaling effectors in breast cancer cells.

Progesterone activation of MAPK was initially reported in T47D breast cancer cells (40). Progesterone stimulation of cells induces cross talk between cytoplasmic PgR-B and ER alpha, which in the absence of estradiol triggers ER alpha/Src association with consequent activation of the Src/Ras/MAPK pathway (40). Activation of MAPK by progestins is needed for the S-phase entry of T47D cells (10). Subsequent studies in *in vitro* reconstituted systems further clarified the molecular mechanism underlying progesterone activation of MAPK cascade by cross talk between PgR-B and ER alpha (41). Such cross talk is also

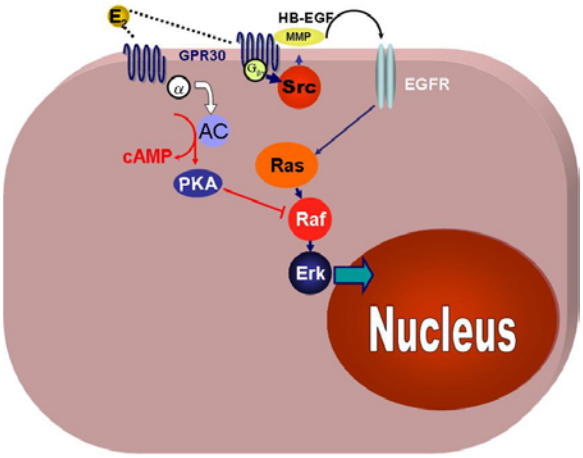

Figure 2. Model of estradiol action through GPR30 and cross talk between cAMP and MAPK pathways in ER-negative breast cancer cells. In ER-negative breast cancer cells, estradiol (E_2) directly binds to GPR30 and induces, through Gβγ-subunit protein activation, a Src-mediated activation of metalloproteinase (MMP) and release of HB-EGF. Transactivation of EGFR then occurs and Erk activation is triggered. Estradiol binding to GPR30 also activates adenylyl cyclase (AC) and increases cAMP levels. PKA activation occurs and Raf is blocked. Erk signaling is then switched off.

responsible for progestin stimulation of endometrial stromal cell proliferation mediated by non genomic pathway activation (73). More recently, it has been shown that activation of MAPK cascade by progesterone through the PgR-B and ER alpha cross talk leads to phosphorylation of histone H3 with the consequent induction of progesterone target genes, thus pointing to the regulatory role of MAPK in the integration between non genomic and genomic signaling activated by steroids (13). Under different experimental conditions, it has been observed that PgR can directly activate Src, without the contribution of ER (64). Rapid activation of MAPK by steroids has been observed in different cell systems, including *in vivo* models (74, 75).

MAPK are also implicated in the ligand-independent activation of ER alpha, as shown by findings demonstrating that activation of MAPK by growth factors phosphorylates and potentiates the transactivation function of ER alpha (76). In addition, expression of constitutively activated MEK-1 in MCF-7 breast cancer cells increases ER alpha-mediated transcriptional activation and accelerates tumor growth *in vivo* (77). Altogether, these data indicate that MAPK pathway can also intersect with steroid receptors at the transcriptional level.

4.4. Integration between cAMP and MAPK pathways in breast cancer

The complexity of signaling pathways, the cross talk between multiple pathways and the presence of feedback loops occurring within the circuits has been actively investigated (78). Integration between different signaling pathways activated by steroid hormones in breast cancer has been explored. As described in the previous section, estradiol treatment of MCF-7 cells triggers activation of PI3-K and Src-dependent pathways with a proliferative final effect. Signaling of steroid hormones can also be regulated by adenylyl cyclase. Traditionally, adenylyl cyclase activity is modulated by receptors that couple to GPRs, and data from different groups have shown that GPR30, an orphan GPR, plays a critical role in steroid signaling (68-69). It binds estradiol and regulates MAPK activation in a transient way, since it is involved in both the rapid activation of MAPK and its subsequent inactivation. These findings indicate that the estradiol control of MAPK axis occurs even in the absence of classical ER. In fact, estradiol treatment of ER-negative cells triggers GPR30 activity that, through Gβγ-subunit protein activation, induces the Src-mediated release of heparin- bound EGF (HB-EGF) from the cell surface. Once released, HB-EGF activates EGFR, which, in turn, triggers MAPK activation (79). A similar pathway, however, can be activated by estradiol occupancy of the classical ER (46 and refs therein). Furthermore in cells lacking ER, estradiol also through GPR30 activation and Gα-subunit protein, stimulates adenylyl ciclase and increases cAMP levels. This event leads to activation of PKA and PKA-mediated block of Raf. In this way, MAPK inactivation follows to the initial MAPK activation (80, 81). Recent work supports such a model. Addition of cAMP in MCF-7 cells activates PKA, which, in turn, phosphorylates the regulatory subunit p85 of PI3-K in serine 83. In this way, cAMP intersects with estradiol by facilitating the binding of ER to PI3-K. This results in a selective increase in Ras/PI3-K association and a net decrease in the Ras/Raf-1 complex. Thus, Ras signaling is mainly channeled to PI3-K rather than to Raf-1/MAPK (82). These data offer an example of how cAMP may act as an inhibitor of MAPK.

The cross talk between cAMP and MAPK signaling pathways is involved in cell transformation. In fibroblasts, elevation of cAMP blocks signaling through the Ras/Raf/MEK pathway and therefore blocks Ras-induced transformation through PKA. Thus, Raf appears to be the major target of PKA in inhibiting signal transmission to MAPK. In this regard, it has been described that elevation of cAMP levels reduces both EGF stimulation of MAPK in MCF-7 cells and the ability of the same cells to form tumors in nude mice (58). Subsequent studies have shown that expression of G protein alpha inhibits the growth of established human tumors of breast cancer cells in athymic mice by inhibiting the MAPK pathway (83). In addition to indicating that interactions between the cAMP and MAPK signaling pathways regulate proliferation of breast cancer *in vivo*, these data also imply that targeting of the cAMP/MAPK axis (i.e. by continuous elevation of cAMP) could be used to block tumor formation.

Figure 2 illustrates the GPR30-mediated actions of estradiol and the cross talk between adenylyl cyclase and MAPK (Erk) in ER-negative breast cancer cells. The initial estradiol activation of Erk is followed by PKA/Raf-mediated inactivation of the same enzyme.

5. SUMMARY AND PERSPECTIVES

To date, most of the studies investigating the non genomic action of steroid hormones have been conducted *in vitro* using cancer-derived cells, and only a small number of these studies concern non-reproductive cells, mainly stromal cells, which strongly contribute to cancer progression. We have to learn much more about the role of steroid-activated pathways as well as their integration *in vivo* with pathways activated by different ligands, such as non-steroid hormones and growth factors. The proteomic approach, in association with the use of animals expressing genetically modified signaling effectors, will be of great help in this complex analysis. Another promising line of research has been initiated by laboratories seeking for ER ligands that preferentially act on the transcriptional or non-transcriptional signaling of ERs. A synthetic compound termed estren mainly induces the non-transcriptional actions of ER, whereas another pyrazole compound induces the transcriptional activity of ER, with minimal effects on its rapid signaling action (84, 85). It is expected that other similar receptor ligands will be found and employed in the study of steroid receptor action as well as in the therapy of receptor-associated diseases.

The emerging field of steroid receptor-mediated signaling activation in breast cancer is very promising and one of the reasons for this mounting interest is offered by the potential use of signalosoma-based approaches to cancer therapy. Recently, new molecules have been identified and used to inhibit the proliferation of breast and prostate cancer cells *in vitro* as well as in immune-depressed mice (20, 45 and submitted). These molecules act at nano-molar concentrations by specifically interfering in the interaction of steroid receptors and Src. They leave unaltered the receptor-mediated gene transcription as well as the signaling transduction that does not depend on steroid receptors. Further investigation is required to validate these approaches to cancer therapy in preclinical and clinical studies and find new strategies to contrast breast cancer.

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Abbreviations: SR, steroid receptor; ER, estradiol receptor, PgR, progesterone receptor; AR, androgen receptor; EGF, epidermal growth factor; HB-EGF, heparin-bound EGF; EGFR, epidermal growth factor receptor; MAPK, mitogen activated protein kinases; MEK-1, mitogen-activated kinase kinase; MMP, metalloproteinase; PI3-K, phosphatidylinositol-3-kinase; GPRs, G protein coupled receptors; PKA, protein kinase A; PKC, protein kinase C.

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