Estrogen receptor signaling as a target for novel breast cancer therapeutics.

Jack-Michel Renoir, Véronique Marsaud, Gwendal Lazennec

Research update

Estrogen receptor signaling as a target for novel breast cancer therapeutics

Jack-Michel RENOIR[1,2,*], Véronique MARSAUD[3] and Gwendal LAZENNEC[4].

[1]U749 INSERM, Institut Gustave Roussy, 94805 Villejuif cedex, France;

[2]University Paris-Sud, Orsay cedex, France;

[3]U1021 INSERM, CNRS UMR 3347, Institut Curie, 91405, Orsay cedex, France;

[4,] INSERMU844, Hôpital Saint-Eloi, 34295 Montpellier cedex 5, France.

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*Corresponding author; phone: 33 (0) 1 4211 4259; Fax : 33 (0) 1 4211 5308

e-mail : jack-michel.renoir@igr.fr

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Abbreviations:

AE: antiestrogen; **AI**: aromatase inhibitors; **AIB1/ACTR/p/CIP/RAC3/TRAM-1/SRC-3:** Amplified in breast cancer 1/Activator of thyroid hormone receptor/p300/CBP-interacting protein/Receptor-associated coactivator 3/Thyroid hormone receptor activator molecule 1/**SRC-3**: steroid receptor coactivator-3; **AKT/PKB**: protein kinase B; **AP1**: Activator protein-1; **BC**: breast cancer; **Bcl2**: B cell lymphoma 2; **CARM1**: coactivator-associated arginine methyl-transferase 1; **DRIP/TRAP**; Vitamin D Receptor Interacting Protein/ Thyroid Hormone Receptor-associated Proteins; **EGF**, Epidermal growth factor; **Erk-1 and Erk-2**: extracellular signal-regulated kinases 1 and 2; **17β-estradiol:** (17β)-estradiol-1,3,5(10)-triene-3,17β-diol; **ER:** estradiol receptor**; FAK**: focal adhesion protein kinase; **Gossypol**: 2,2'-bis-(Formyl-1,6,7-trihydroxy-5-isopropyl-3methylnaphtalene; **HER/Erb-B**: human epidermal growth factor receptor/avian erythroblastosis oncogene B; **IGF**: Insulin growth factor; **IRS1**: Insulin receptor substrate 1; **LSD1/KDM1A**: lysine-specific histone demethylase 1; **MAPK**, mitogen activated protein kinase; **MIBE**: ethyl 3-[5-(2-ethoxycarbonyl-1-methylvinyloxy)-1-methyl-1H-indol-3-yl]but-2-enoate; **mTOR**: Mammalian target Of Rapamycin; **NR**: nuclear receptor; **PAX-2**: Paired Box-2 gene product; **PDK1:** 3-phospho-inositide-dependent protein kinase; **PELP1/MNAR** proline-, glutamic acid-, and leucine-rich protein 1/Modulator of the non-genomic action of nuclear receptors; **PI3K** : phosphoinositide-3 kinase; **PR**: progesterone

receptor; **PRMT1**: protein arginine methyltransferase; **PTEN**: phosphatase and TENsin homolog; **PTM**: post-translational modification; **Src**: sarcoma virus tyrosine kinase; **SERM**: selective estradiol recetor modulator; **SERD**: selective estradiol receptor downregulator; **SDF-1**: Stromal cell-Derived Factor-1; **SHARP**: SMRT/HDAC1 associated repressor protein; **SMRT**: silencing mediator for retinoid and thyroid hormone receptor; **SP1**: Specificity protein 1; **SWI/SNF**: SWItch/Sucrose Nonfermentable chromatin remodeler; **TRAP220**: Thyroid receptor-associated protein complex 220 kDa component; **uPA**: urokinase-type plasminogen activator.

Abstract

In breast cancer (BC) epithelial cells the mitogenic action of estradiol is transduced through binding to two receptors ERα and ERβ which act as transcription factors. Antiestrogens (AEs) and aromatase inhibitors (AIs) are used clinically to arrest estrogen-dependent growth of BC. In the case of AE or AI resistance, therapeutic benefit is gained by inhibiting growth factor receptors with Herceptin and lapatinib. Estrogen effects are mediated not only through nuclear ERs but also through cytoplasmic/membrane ER and G-protein-coupled ER. These estrogen-binding systems associate with various proteins affecting cell cycle signaling, proliferation and survival. The partners of nuclear ER include SRC1-3, HDACs and ERβ itself, but also newly identified proteins, such as E6-AP, LKB1, PELP1, PAX-2 and FOXA1. The partners of extranuclear ERα include PI3K and the tyrosine kinase Src. These various factors are all potential targets for therapeutic intervention. In addition, BC proliferation is enhanced by insulin and EGF which stimulate signaling through the MAPK and PI3K/AKT pathways by activation of IGF-1R and EGFR axes, respectively. These pathways are tightly interconnected with ER-activated signaling and indeed, membrane ERα complexes with Src and PI3K. Chemokine-mediated signaling also modulates the estrogen response. Inhibiting these pathways with specific inhibitors or activating some of them by gene manipulation, may be therapeutically valuable for arresting BC cell cycle progression and inducing apoptosis in order to antagonize hormone-resistance. Here, we review some newly

identified putatively targetable ER partners, and highlight the need for developing tumor-targeting drug carrier systems affecting both the tumor cells and the tumor environment.

I. Introduction

Estrogen receptors (ERs) belong to the subfamily of ligand-regulated transcription factors which transduce hormones signaling into a large variety of physiological responses in various organs [1]. The two structurally related ERs namely ERα and ERβ are the products of two separate genes differentially distributed in tissues. ERα is responsible for estrogen-induced mitogenic signaling in epithelial cells in breast, uterine and ovarian tissues [2]. In the normal mammary gland, estradiol (E_2) binds to ERα and ERβ, which controls cell proliferation and differentiation [3]. Both ER isoforms are produced in similarly low amounts in the normal breast, whereas more ERα than ERβ is expressed in breast cancer (BC) cells. Importantly, ERα is the only ER that is detected by immunohistochemistry in BC biopsies. Only tumors with nuclear-free ER cells are classified as "ER-negative". At least 70% of BCs are ER-positive (ER^+) and express mainly ERα or progesterone receptor (PR) or the erythroblastosis oncogene-B2 (ErbB-2, HER2/NEU) or all three. ErbB-2 is a member of the HER family of trans-membrane receptor tyrosine kinases (RTK), which also includes the epidermal growth factor receptor (EGFR/HER 1).

Patients with ER- and PR-positive BC are currently treated with hormone therapy (HT) to inhibit ER signaling. HT uses two approaches: antagonizing agonist ligands binding to ER with antiestrogens (AE) or blocking E_2 synthesis with aromatase inhibitors (AIs). Despite the high level of success of HT, many BCs acquire resistance. Some tumors only express Erb-B2

and do not respond to HT; in such cases the use of trastuzumab (Herceptin) a humanized monoclonal antibody targeting ErbB-2, has offered a considerable benefit, but a significant number of breast tumors fail to respond [4]. ER and ErbB-2 have been the targets of choice for BC treatment over recent years. However, some tumors, classified as triple negative [5], do not express any ER, PR and ErbB-2, and consequently are resistant to HT and trastuzumab. Triple negative BCs are actually considered as cancers completely different from hormone-dependent BCs. Their prognosis is poor and they are currently treated with chemotherapy (i. e. Paclitaxel). Understanding of the molecular mechanisms implicated in the development of these different malignancies, was improved by both clinical and fundamental research over the past decades. However, despite the progress made in our understanding of these diseases, as well as in the discovery of new treatments, the number of patients dying from BC has not decreased substantially. There is no doubt that new effective therapies are required. One difficulty is the lack of specific markers that can be used to distinguish malignant cells from normal cells. Indeed, current treatments simply target over-expressed factors such as ER and ErbB-2. Deciphering the mechanism of action of estrogens through the transcription activity that they trigger following binding to their cognate receptors has led to the identification of many new actors. This prompted the pharmaceutical industry to search for new inhibitors that can be used in BC treatment, and there are consequently numerous clinical trials underway combining several molecules. Most of them affect the regulators of post-translational modifications of ER, including phosphorylation, acetylation, prenylation and ubiquitination.

Moreover, a small pool of ER localizes in the cytoplasm and at the membrane tightly bound to adaptor proteins, forming multiprotein complexes that trigger the activation of the MAPK and AKT pathways. This also has led to envisage the use of new inhibitors. In this

review we will analyze some of the factors modulating the effects of estrogens on ER, which could serve as new targets for the treatment of both estrogen-sensitive and insensitive breast tumors.

II. Estradiol receptors function and endocrine therapy in breast cancers

Like all other members of nuclear receptors (NR) family, ERs are activated through either agonist ligand binding or phosphorylation at various sites or both (see [6] for a review). The ER proteins are generally believed to shuttle between the cytoplasm and nucleus and *in vitro* experiments have demonstrated that ligand-free ERα, like other steroid NRs, is maintained in a non-DNA binding form in a multi-chaperone complex organized around Hsp90 (review in [7]). Little information is available with regard to ERβ but both ERs are believed to similarly activate gene transcription upon classical estrogen binding. ER-mediated transcription is a highly complex process involving multiple coregulatory factors and "cross-talks" between different signaling pathways (**figures 1 and 2**). These mechanisms have been described in detail in other reviews and therefore are only briefly summarized here (for more details see [8]).

II. A. Canonical genomic ER-mediated transcription mechanism

In response to estradiol binding, ERα undergoes conformational changes, which control its interaction with heat shock proteins (although the interaction between ERβ and Hsp90 is poorly documented) and coregulators; these interactions determine ER binding to the 13bp estrogen response element sequence (ERE) within the promoter (**figure 1**). ER-dimers dynamically and sequentially recruit various regulatory protein complexes

contributing to chromatin remodeling, thus strongly enhancing transcription activity [9]. The NR coactivators identified with ER include the general transcription factor p300/CBP. P300/CBP is ubiquitously expressed and serves as coadaptor between NRs and DNA. It has a critical role in cell cycle regulation, cell differentiation and apoptosis and has histone acetyltransferase (HAT) activity [10, 11]. Importantly, HATs are required for full ER-mediated transcriptional activation. P300/CBP also interacts with other HATs such as PCAF [12], and acetylates components of the basal transcription machinery. Methyl-transferases including CARM1 and PRMT1 are also ERα-associated coactivators. Members of the p160 proteins family, namely steroid receptor coactivator 1 (SRC-1), SRC2 and SRC3 (ACTR, RAC-3, pCIP, TRAM-1, AIB1 encoded by the *AIB1* gene), play various roles in the recruitment of the pre-initiation complexes DRIP/TRAP [13].

E_2-ERα complexes affect the transcription of genes involved in proliferation, differentiation, survival, and, particularly relevant for cancer, in the stimulation of invasion, metastasis and angiogenesis. Among these genes, some are activated like those involved in cell cycle progression (such as *c-myc, cyclins D, A and E)* and the expression of others, such as the gene for the cyclin-dependent kinase (CDK) inhibitor $p21^{Waf1/Cip1}$, is decreased [14]. Consequently, the growth of ERα-expressing (ER^+) cells from breast tumors is E_2-dependent and the removal of E_2 leads to regression. Therefore, ERα is a well-established predictive marker of hormone sensitivity and a positive prognostic marker in BC, identifying tumors for which endocrine treatment is likely to be effective. The presence of ERβ inhibits both ERα-mediated transcription and E_2-induced proliferation in various cancer cells [15-17]. Therefore, ERβ in BC lesions is thought to be associated with more benign tumors. Both ERα and ERβ may also be found in endothelial cells and vascular muscles, concomitantly with ER

variants (see below). Furthermore, ERα and ERβ differentially regulate both the proliferation and apoptosis of normal mammary epithelial cells [18]. It is currently believed that the ERα/ERβ ratio is a key element in the regulation of E_2 activity in BC cells [19].

Ligand-activation of ER may also stimulate indirect binding of ER to DNA by protein-protein interactions with transcription factors such as AP-1 or Sp-1, which anchor the pre-initiation complex to ERE. For both direct and indirect association of ER with DNA, recruitment of co-activators modulates gene activation and subsequent protein production [8].

ERs are phosphorylated at multiple sites by a variety of kinases. Such phosphorylation may result from both estrogen binding to ER inducing activation of various growth factor receptors or other kinases [6]. Phosphorylated ERα binds directly or indirectly to DNA, recruits co-activators and triggers transcription (**figure 1**). Importantly, ER-mediated transactivation can reach its maximal level only if ER is phosphorylated, even in the absence of E_2 binding.

Various ER variants may alter the estrogenic response. This is the case of ER46, an abundant N-terminal (A/B)-deleted ERα splice variant and highly effective transducer of membrane initiated responses in endothelial cells. ER46 participates in the rapid stimulation of the vascular endothelial nitric oxide synthase (eNOS) and leads to E_2-ER-mediated vasodilatation. These effects on tumor vasculature through ER in endothelial and stroma cells may explain the AE-mediated anti-tumor activity in ER-negative BC xenografts [20, 21]. ERα-36, an ERα variant lacking the A/B N-terminal domain and a truncated ligand-binding C-terminal domain, has been implicated as a mediator of extra nuclear (non-genomic) actions.

II.B. The non-genomic pathways

II.B.1 Membrane ER

It has long been established that E_2 induces rapid effects emanating from the membrane. Various E_2-induced signaling cascades have been identified in the extra nuclear compartment (non-genomic mechanism), and involve direct interactions of a small pool of ER (principally ERα) localized at the membrane (mbER) with other proteins. Indeed, ERα is found in multiprotein complexes that include growth factor-dependent kinases and adaptor proteins [22] (**figure 2**). In addition, mbERα binds in a ligand-dependent manner to the p85α regulatory subunit of PI3K [23]. Palmitoylation enables mbERα to interact with caveolin-1. Caveolin-1 gene inactivation promotes increased ERα expression and up-regulation of cyclin D1 [24]. Binding of E_2 to mbER complexes leads to de-palmitoylation and dissociation of ERα from caveolin-1, then to the activation of many downstream signaling events such as the tyrosine kinase Src, the p85 PI3K subunit, MAPK, AKT, p21ras and protein kinase C, helping ERα to move to other membrane micro-domains [25]. Non-genomic functions resulting from E_2-binding to mbERs affect cell proliferation and survival (ERα) and apoptosis (ERβ) [26].

II.B.2 GPER

Estrogen also signals through a seven trans-membrane G-protein-coupled receptor (GPCR-30) and E_2-GPCR-30 complexes (**figure 2**) activate Erk-1 and Erk-2. Despite alternative suggestions to attribute the non-nuclear effects of E_2 to ERα36 and not to GPCR-30 [27], a significant amount of evidences has established the function of GPCR-30 as membrane ER with specific binding characteristics (see [28] for a review). Indeed, E_2 acts as an agonist towards GPCR-30, but ER antagonists (both mixed and pure) can also act as agonists, similarly to a variety of phyto- and xenoestrogens which stimulate cAMP production (**figure**

2). This receptor now named GPER-1 (G-protein-coupled ER-1), stimulates adenylcyclase and the cAMP-mediated regulation of EGF-MAPK axis [29]. Conversely, GPER is up-regulated by EGF in ER-positive BC cells; moreover, GPER was suggested to act as an inducer of ERα-36 expression in various BC cells including the "ER-negative" cell line (MDA-MB-231). These and other diverse findings demonstrate the tight interplay between ER- and EGFR-signaling, and illustrate the complexity of estrogen action in BC cells. This complexity is exemplified by the differential activity of ER ligands towards GPER; GPER antagonists with ER have been identified, such as G15 and G36 [30] and MIBE [31] **(figure 3)**. They are all promising molecules capable of inhibiting both the effects of estrogens acting as inducers of ER-mediated transcription and also those emanating from the membrane of BC cells.

II.C. Hormone therapy

Numerous reviews have already thoroughly described the various advantages and disadvantages of the use of antiestrogens and aromatase inhibitors. We will only present a brief summary here.

II. C. 1. Anti-estrogens

Two distinct classes of synthetic AE have been developed to treat $ER^+/PR^+/Erb\text{-}B2^-$ tumors (**figure 3**). Selective Estrogen Receptor Modulators (SERMs) are a class of ER ligands, exemplified by tamoxifen (Tam, Nolvadex) and raloxifene, that act as either AE or agonist depending on the tissue and the cellular promoter context. Tamoxifen has been in clinical use for over 30 years and is metabolized in the liver to 4-hydroxy-Tam (4-OHTam), which has a 100 X greater affinity for ERα than tamoxifen does [32]. The Selective Estrogen Receptor Downregulators (SERDs) are a class of steroidal, pure AEs, which are devoid of any agonistic

activity in any tissue [32]. Faslodex® (fulvestrant, ICI 182780) is currently the only SERD in clinical use, and it is employed in case of Tam resistance. Similar to the other SERD, RU58668, Faslodex® exhibits a dual mode of action: first it binds to ER and thereby induces the formation of an inactive complex, blocking ER dimerization and nuclear localization, and second, it targets ERα to ubiquitination prior to its degradation by the proteasome. These effects are accompanied by the inhibition of ER-mediated transcriptional effects [33]. However, after arrest of AE treatment, the inhibitory effects of AEs, including SERDs, are reversed by estrogens such that the efficacy of the drugs is limited, resulting in a limited efficacy of the drugs [34].

Tamoxifen, the first therapeutic hormone antagonist or antihormone in clinical use, reduces BC progression and is effective in inducing the arrest of tumor progression in 50% of patients. However, the response to HT is transient, and relapse of treated women often occurs with a median duration of 20 months [35], despite the persistant expression of ER. Many hypothesis may explain hormone therapy-acquired BC resistance including the expression (or loss) of inactivated (or truncated) ER isoforms, increased activity of coactivators or other transcription factors such as AP1, post-translational modifications e. g. phosphorylation and methylation, increase tyrosine kinase signaling of membrane EGF and IGF receptors (see ref in reviews [6, 35-38]). The activation of the growth factor receptors, implicated in PI3K/AKT and Erk pathways and leading to deregulation of cell cycle and to apoptosis has certainly a major role to play in HT resistance [39, 40] (see below). Another attractive target possibly involved in SERM-acquired resistance is the antiestrogen binding site (AEBS), a site supposed to be located on the ER molecule [41], but recently characterized to be formed by heterooligomerisation of two enzymes, the 3-β-hydroxysterol-Δ^8-Δ^7-

isomerase and the 3β-hydroxysterol-Δ^7-reductase [42]. These enzymes are involved in post-lanosterol cholesterol biosynthesis. Tamoxifen, raloxifene and other SERMs, in contrast to SERDs inhibit the AEBS, leading to the accumulation of specific sterols and to apoptosis and autophagy in MCF-7 BC cells [43]. Specific AEBS ligands (e.g., DPPE (N-diethyl-2-[4-(phenylmethyl) phenoxy]ethanamine)) and analogs are in Phase III clinical trials in combination with doxorubicin with encouraging results in metastatic BC [44, 45].

II. C. 2. Aromatase inhibitors

Approximately 50% of patients with advance disease do not respond to first-line treatment with Tam and almost all patients with metastases relapse and die from the disease [35]. A second endocrine therapy strategy has emerged consisting of the use of AIs to reduce the production of estrogen in peripheral tissues and within the tumor. Aromatase converts androstenedione into androgen, then to estrone and E_2. Aromatase is expressed in many endocrine tissues including BC cells. Therefore, selective AIs have been designed to reduce circulating estrogen levels. Blocking E_2 production is considered an option for premenopausal women with ER-positive tumors. In postmenopausal women, the ovary is no longer the primary source of E_2 which is produced in the fat tissues, and AIs have been widely successfully used in the treatment of postmenopausal women with advanced BC. Indeed, anastrozole and letrozole but not exemestane (**figure 3**) exhibit stronger anti-proliferative activity than Tam in patients with ER-positive tumors, and this treatment can also be used to reduce the side effects of Tam. Additionally, AIs are also useful for treating AE-resistant BC [46].

III. Growth factor signaling

III. A. EGF pathways

Most BC cells express receptors for peptide growth factors, such as EGF. These tyrosine kinase receptors are activated following binding of these peptides in their extracellular domain. In the case of EGF receptors ErbB-3 and ErbB-4, EGF binding induces the formation of receptor homo- and hetero-dimers with ErbB-2, leading to enhancement of the receptors' kinase activity (except in the case of dimers with Erb-B3 which, in contrast to the other members of the EGFR family, is devoid of TK activity). This binding ensures the auto-phosphorylation of the receptors and initiates a cascade of downstream signaling. The consequences of EGFR activation are multiple and include cell proliferation (via activation of the Ras/Raf/Mek/MAPK pathway), cell cycle progression and survival (via activation of the PI3K/AKT pathway) and expression of various genes encoding proteins such as VEGF. No ErbB-2 ligand has been identified but the ErbB-2/ErbB-3 dimers can be activated by the peptides derived from heregulin (HRG) which are ligands for ErbB-3 only. The binding of HRG to the ErbB-2/ErbB-3 hetero-dimer activates the ErbB-2 TK activity, leading to a mitotic response and the induction of anti-apoptotic Bcl-2 family members including Mcl-1 [47]. In human BCs, an increase of Erb-B2 expression is associated with an increase of SRC-1 and SRC-3 [48].

III.B. Insulin-like growth factor signaling

The binding of IGFI and IGFII to insulin-like growth factor receptor 1 (IGF-1R) activates intracellular pathways that regulate cell growth and survival control (**figure 2**). IGF-1R is a trans-membrane receptor with TK activity. IGF-1R functions as a homo- or heterodimers with the insulin receptor I. IGF binding to IGF-1R triggers two different (although inter-connected) pathways. The recruitment of a docking specific intracellular receptor substrate (IRS-1) at the phosphorylation sites transduces the signal to the shc/Ras/raf/MAPK pathway, leading to

accelerated cell proliferation. IGF I and II are secreted in practically all epithelial cells, and the binding of IGFI to IGF-1R increases tumor growth and development. Furthermore IGF-1R and the insulin receptors (IRα or IRβ) can hetero-dimerize and transduce the signals triggered by insulin, a mechanism associated with a poor prognosis [49]. Although there are no direct interactions between ER proteins and IGF-1R, there is evidence that IGF-1R maintains cell-induced proliferation and anti-apoptotic activity even in the presence of AE. In response, IGF-1R is phosphorylated by PI3K, leading to AKT recruitment at the membrane and consequently to its activation through phosphorylation either on ser473 by the Ric/mTOR complex or on thr308 by PDK1. Downstream from AKT, mTOR is also involved in the regulation of cell cycle proteins. The activation of this second branch of IGF-signaling is critical for cell cycle progression and survival; indeed, it has been clearly demonstrated that inhibition by phosphorylation of pro-apoptotic molecules such as the Bcl-2 family member BAD and the cleavage (activation) of caspase-9, led to suppression of apoptosis (reviewed in [50]).

IGF-1R is over-expressed in the majority of BCs (90 to 95%) and is often co-expressed with ER. Moreover, estrogens induce the expression of IGF-1R and IRS-1, thereby reinforcing the IGF-induced responsiveness of BC and Tam resistance. IGF- and ERα-regulated pathways are thus intricately interconnected, in mammary development and BC. High circulating plasma concentration of IGF-1 is a marker for an increased risk of relapse under treatment with adjuvant Tam. A number of small chemical inhibitors and antibodies targeting IGF-1R inhibitors have been developed the most advanced in clinical trials being OSI-906 (Linsitinib) and BMS 754807 (**figure 4**).

IV. Resistance

Whatever the endocrine treatment used, resistance may occur. This is especially true with Tam which is never given for more than five years. Moreover, patients whose tumors overexpress ErbB-2 (15-20% of all BCs) are resistant to endocrine treatment. The molecular causes of endocrine resistance are incompletely understood. ER- and PR-negative menopausal BCs over-expressing Erb-B2 are currently cured with two FDA approved treatments: Trastuzumab (Herceptin) and the small chemical molecule tyrosine kinase inhibitor lapatinib. Trastuzumab binds to an epitope in the juxtamembrane region of the ErbB-2 receptor. This binding induces uncoupling of ligand-independent HER2-HER3 heterodimers and inhibition of downstream signaling. Binding also causes antibody-dependent, cell-mediated cytotoxicity. Although many BCs with *HER2* gene amplification respond to trastuzumab, a significant fraction of these subsequently progress. Several mechanisms of resistance to the antibody have been reported; these mechanisms include enhanced signaling by RTKs, amplification of PI3K signaling as a result of mutations in this pathway, and the presence of truncated forms of Erb-B2 devoid of the antibody-binding epitope in the receptor's ectodomain. A recent study demonstrated that exposure of ER-positive BC cells to fulvestrant increased the expression of ErbB-3 and/or ErbB-4 and sensitivity to their potent ligand heregulin although these effects are depended on the cell line tested [51]. This observation severely compromises the use of fulvestrant in first line hormone therapy because BC cells may be able to compensate for the growth-inhibitory effects of fulvestrant by growth stimulation via ErbB-3-4 [52]. It remains to be determined whether this type of fulvestrant-associated increase of ErbB-3-4 activity can occur with other AE, particularly RU 58668, another pure AE which counteracts fulvestrant-acquired resistance in xenograft models [53]. The Erb-B2 TK inhibitors (TKI) lapatinib (a dual inhibitor of Erb-B1 and Erb-B2 TK function) and neratinib have clinical activity as single agents or in

combination with chemotherapy in patients who relapsed under trastuzumab [54]. These findings suggest that trastuzumab-resistant tumors continue to depend on the TK activity of Erb-B2, requiring the combination with TK activity or targeting of another pathway. Unfortunately in case of triple negative breast cancers there is no current treatment available to provide a good outcome.

All BCs express EGFR (**figure 2**) which regulates cell cycle and anti-apoptotic signaling. Many mechanisms other than ErbB-2 may explain Tam-acquired resistance including the deregulation of receptor expression or maturation. The deregulation of post translational modifications of both ERs and their cofactors has been highlighted. Also, increased and deregulated cell cycle and apoptosis signaling are certainly among the major causes of resistance [40]. In BC over-expressing Erb-B2, the concomitant over-expression of SRC-3 contributes to trastuzumab resistance by activating IGF signaling and to Tam resistance by increasing the agonistic activity of this SERM [48].

Cetuximab (Erbitux) is a humanized monoclonal antibody against EGFR used in the treatments of colorectal cancers. Cetuximab has been assessed in combination with TK inhibitors (such as erlotinib) (**figure 5**) for treating patients with ER^+ BC, but the responses were not encouraging. However, new molecules inhibiting the HER members by competing with their ligands may be of therapeutic value, particularly in combination with drugs targeting the Erb-B2 receptor network. A combination of this type is undoubtedly required for better inhibition of this pathway and, hence, improved clinical activity. In support of this view, lapatinib is a dual inhibitor of EGFR and Erb-B2 and in combination with paclitaxel has exhibited good efficacy in the treatment of women with Erb-B2-positive BC [55].

V. Potential new targets

V.A. Co-activators and corepressors

V.A.1. SRC1-3

Among coactivators which have been identified as robust enhancers of ER-regulated transcription, SRC-1 and SRC-3 are frequently over-expressed in BC tumors in association with enhancement of ErbB-2, a status associated with poor survival. SRC-1 serves as a general transcription enhancer for many transcription factors, and SRC-3 over-expression participates in positive cross-talk with both IGF-1 pathway and AE resistance (see [48] and ref herein). SRC-3 has also been identified as a mammary tumor-initiating factor and SRC-3$^{-/-}$ mice are defective for oncogene- and carcinogen-induced BC initiation, and for metastasis [56]. In BC cells over-expressing ErbB-2, SRC-3 participates in the action of trastuzumab treatment through activation of the IGF signaling [57]. These various observations indicate that the ability to abolish SRC-1/3 activities would be valuable additions to the established arsenal of targeted therapies for BC, particularly in overcoming resistance. O'Malley et al. have been searching for inhibitors of these coactivators and recently found that gossypol (**figure 6**), a natural product from cottonseed, disrupts interaction between NR and SRC-3 and down-regulates SRC-3 not only in BC cells but also in lung, prostate and liver cancer cells [48]. Gossypol was described a long time ago as a male infertility molecule and was considered for use in male contraception. Gossypol binds to Bcl-2 and Bcl-XL and antagonizes their anti-apoptotic activities. Thus, gossypol represents the prototype of a new class of potent anticancer molecule that could be used in combination with other chemotherapeutics to fight resistance in cancers. Consequently, phase II-III clinical trials to assess the value of gossypol in several types of cancer are currently underway (http://www.clinicaltrials.gov).

V.A.2. HDACs

Five lysines on ERα are reportedly acetylated by p300: Lys266, Lys268, Lys299, Lys302 and Lys303, all localized in the hinge region. Other PTMs of ERα may affect the same lysine residues, but with different consequences on BC cell behavior. This is the case of Lys302 which in addition to acetylation can be ubiquitylated, sumoylated or methylated [6]. The effects of ERα acetylation result from a two-step mechanism: short exposure of cells to HDAC inhibitor (HDACi) leads to acetylation and stabilization of the receptor (as well as of that of p300/CBP), whereas after long exposures, the receptor is delocalized and subsequently degraded by the proteasome [58]. By contrast, exposure to HDACis of ERβ-containing BC cells and ERβ-rich ovarian cancer cells stabilizes ERβ isotype [59]. HDACis block cell cycle and induce apoptosis in various cancer cells. Thus, several phase I and II clinical trials are currently underway with these anticancer agents. In breast tumor models, several HDACis exhibit antiproliferative effects in vivo. Importantly, restoration of ERα expression was observed in ER-negative BC cells following exposure of cells to pan HDACis, a process potentiated by the DNA methyl transferase inhibitor 5-aza-deoxycitidine [60]. When HDACs are inhibited, a decrease of EGFR mRNA in ER-negative MDA-MB-231 cells is observed and in vivo; concomitantly, a re-sensitization of these cells to Tam is observed, strengthening the potential usefulness of HDACis combined with AE for BC treatment [61].

HDACis are promising anticancer drugs because they have multiple targets in cancer cells [62]. HDACis activate the acetylation process and inhibit tumor growth through the repression of oncogenes, including *c-myc,* but they also activate tumor suppressors such as *CDKN1A* encoding the CDK inhibitor $p21^{WAF1/CIP1}$ [63]. HDACis inhibit cell cycle and activate programmed cell death, differentiation and angiogenesis in many cancer cells and in animal

models [62]. Some HDACis have already been approved by the FDA (SAHA or "Vorinostat"; CG1511 or "Belinostat", LBH589 or "Panobinostat") and many (**figure 7**) are in clinical trials for BCs (NCI clinical protocol NCT007777049; see http://www.cancer.gov). Importantly, inhibitors of the class II HDACs like Etinostat (MC1575) contrary to TSA do not decrease ERα expression but enhance expression of ERβ without induction of apoptosis. This is accompanied by up-regulation of $p21^{waf1/CIP1}$ gene and antiproliferative effects [64]. This type of HDAC inhibitor could be of therapeutic value mainly in association with other drugs, for example ERβ agonist ligands, TKIs or HSP90 inhibitors (see below).

Another potentially exploitable target in BC is the microtubule-associated HDAC-6 which can deacetylate Hsp90. Specific inactivation of HDAC6 by HDAC inhibitors results in acetylation of Hsp90 leading to dissociation and proteasome-mediated degradation of client proteins, and subsequent cell death. The G protein-coupled receptor kinase 2 (GRK2) is a key modulator of HDAC6. GRK2 phosphorylates HDAC6 leading to α-tubulin deacetylase activity that regulates key cellular processes dependent on cytoskeletal rearrangements such as migration, polarity and cell spreading [65]. Therefore, it is plausible that inhibiting HDAC6 deacetylase activity could be therapeutically beneficial against BC metastasis. However, specific inhibitors of this type of HDAC are yet to be developed.

V.A.3. PAX-2

High levels of SRC-3 (AIB-1) and ErbB-2 have been described in aggressive BC and more recently, the laboratory of J.S. Carroll demonstrated that the Paired Box-2 gene product (PAX-2) is a critical Tam-recruited transcriptional repressor of the *ErbB2* gene [66]. Elevated AIB-1 expression can lead to competition with PAX-2 binding of Tam-ER complex to DNA, directly resulting in increased ErbB2 protein expression. PAX-2 is generally described to

be a transcriptional activator with a tissue-specific activity, acting as a repressor in BC and, a determinant of SERM action in female reproductive tissues [66].

V.A.4. FOXA1

The Forkhead protein (FOXA1/HNF3α) plays a determinant role in the transcriptional activity of E_2-ERα complex, modulating ERα-chromatin interactions and thus, the endocrine response of BC cells [67]. FOXA1 is negatively regulated by the CCCTC-binding factor (CTCF), an upstream regulator of FOXA1-chromatin interactions. FOXA1 is required for E_2 and Tam action in E_2-responsive BC cells. Moreover, FOXA1 helps in reprogramming ERα binding to gene promoters in tumors from patients with drug resistant BCs at different sites than those at which ERα binds in tumors from Tam-sensitive patients. FOXA1 is absolutely required for ERα-binding to promoters even in the absence of ER-ligand binding [68]. As a consequence silencing of FOXA1 may be of therapeutic value.

V.A.5. E6-AP

E6-associated protein (E6-AP) is an E3-ubiquitin ligase which functions as a coactivator of steroid hormone receptors including ERα [10]. The abundance of E6-AP in BC tumors is inversely correlated to that of ERα. In transgenic mice that over-express the ubiquitin-ligase E6-AP, E_2 failed to initiate mammary tumor development, whereas such tumors develop rapidly in mice that over-express an inactive E6-AP mutant. Together with the strong inverse correlation between survival and expression of E6-AP, these findings suggest that E6-AP may act as a tumor suppressor [69]. In addition to its utility in diagnosis, gene amplification of E6-AP could be of potent useful.

V.A.6. Methyl transferases

Transient methylation of ERα on arg260 by PRMT1, a coactivator of numerous NRs, has been shown to participate in the exclusive cytoplasmic localization of the receptor and

to mediate its extranuclear function by triggering its interaction with the p85 subunit of PI3K and Src [70]. As a result of this process, AKT is phosphorylated, activating the downstream cascade to induce rapid events leading to non-genomic effects of E_2. Thus, PRMT1 contributes to the regulation of E_2-induced non-genomic downstream effects. The FAK adhesion protein, a substrate of Src also interacts with arg260-methylated ERα [6]. It is possible that BC cells with methylated ERα may be involved in migration and metastasis. Consequently, targeting PRMT1 through specific inhibitor (such as the water soluble AMI-1, **figure 6**) or siRNAs could decrease this property for a better therapeutic success. However, no data have been obtained in in vivo experiments with this type of PRMT1 inhibitors.

The synergistic activities of HDAC inhibitors with those of methyl transferase inhibitors led to the finding that pargyline, an inhibitor of the lysine-specific demethylase 1 (LSD1/KDM1) increased acetylation of the specific LSD1 substrate H3K4 and enhanced methylation of histone acetylation H3K9 [71]. Additionally, LSD1 inhibitors participated in reexpression of aberrantly silenced genes [72]. Thus combined treatment with pargyline and SAHA resulted in synergistic reexpression of genes including those that encode critical nuclear transcription factors, which mayresult in the following: i) an induction of apoptosis and a reduction of migration of BC cells following their translocation from the nucleus to mitochondria ([71] and ii) an induction of growth inhibition. The possibility of these combinations synergizing with either antiestrogen or aromatase inhibitors may represent a promising epigenetic approach for BC treatment. Importantly, LSD1/KDM1A is enriched in BC [73] and interacts with ERα [74] through the coactivator proline-, glutamic acid-, and leucine-rich protein 1 (PELP1/MNAR) [75, 76], forming an axis connected with Erb-B2/HER pathway. PELP1 is deregulated in several hormone-responsive malignancies including breast tumors [74] and its elevated expression correlates with poor prognosis [77]. Moreover,

PELP1-LSD1 positively regulates Erb-B2/HER2-aromatase [75] and the TK activity of Erb-B2 regulates aromatase acytivity [78]. As a consequence, inhibiting the LSD1/PELP1-Erb-B2 signaling represents a novel strategy to circumvent hormone resistance in breast cancer [79, 80]. However, despite FDA approval, the broad target spectra of pargyline imposes careful administration in patients in order to avoid side effects, and that could be attained through the use of nanocarriers loaded with these drugs as shown in [79].

V.A.7. LKB1/AMPK

The gene *LKB1* (liver kinase B-1) encodes a calcium-calmodulin regulated Ser/Thr kinase which mainly phosphorylates the members of the AMPK family and is considered a tumor suppressor. Phosphorylation of LKB1 activates AMPK which itself participates in the downstream inactivation of mTOR, leading to cell proliferation arrest and apoptosis control. The LKB1/AMPK complex regulates positively cell energy metabolism, and negatively cell cycle progression in various cells. In BC cells, weak expression of LKB1 is associated with high grade tumor. Over-expression of LKB1 blocks BC cell proliferation in G1 in a p21- and p53-dependent manner [81] and arrests migration and invasion through inhibition of metalloproteinases MMP-2 and MMP-9. Expression of LKB1 also negatively regulates angiogenesis through decreasing VEGF and bFGF expression and thus weak vascularization [82]. Moreover, LKB1 interacts with PTEN and with the Brg1 protein encoded by the Brahma-Related-Gene1 *Brg1*, a component of the SWI/SNF chromatin remodeling complex (for a revue see [83]). These findings suggest that LKB1 is a tumor suppressor and low LKB1 expression in BC patients is linked to a poor prognosis [84]. ERα was proposed to act as a repressor of LKB1. However, LKB1 was found to directly interact with ERα in the nucleus of MCF-7 cells, functioning as a co-activator to enhance E2-induced ERα-mediated transcription [85]. This finding was inconsistent with that of a tumor suppressor. Additional studies have

found that the LKB1 promoter contains several EREs and that ERα represses LKB1 expression [86]. E2 up-regulates LKB1/mRNA levels decreasing ERα expression in MCF-7 cells.

Thus, LKB1 may be considered a potential therapeutic target for BCs by mediating ERα through a negative transcription loop. This assumption is reinforced by the fact that the AMPK activating drug, metformin, used in the treatment of diabetes of type II (insulin-independent), decreases aromatase expression in BC cells [87] and consequently the plasma E2 concentration. In general, stimulation of LKB1 leads to inhibition of cell adhesion, invasion and migration following AMPK activation and suppression of mTOR [88]. Although no specific small molecule activators of LKB1 are available, approaches involving manipulation of *LKB1* gene expression deserve attention.

V.A.8. HOXB7 proteins

Homeobox genes express nuclear proteins which act as transcription factors during normal development and differentiation. One of the Homeobox genes *HOXB7* was shown to be an ERα-responsive gene that is significantly over-expressed in Tam-resistant MCF-7 cells and in patients with distant metastasis [89]. This elevation of HOXB7 protein has been directly linked to the acquisition and maintenance of SERM resistance [90]. Thus, antagonists of HOXB7 may be important tools to circumvent Tam resistance; these antagonists are not yet available but the incorporation in nanocarriers of siRNA targeting HOXB7 warrants to be evaluated in appropriate xenograft models.

V.A.9. TLE1

The transducin-like enhancer protein 1 (TLE1) is another modulator of the transcriptional activity of ER. In particular, combining the chromatin-immunoprecipitation (ChIP) technique with high throughput sequencing Carroll et al. found a significant overlap of TLE1 binding sites in MCF-7 cells with ER targets [91]. Among these genes some are directly

involved in cell division and can be down-regulated by the transfection of TLE1 siRNAs. These data support the therapeutic use of siRNA for modulating TLE1-ER interaction.

V.A.10. The intriguing role of ERβ

ERs are widely distributed in the body. ERα is mainly expressed in uterus, prostate (stroma), breast (luminal cells), ovary (theca cells), bone, epididymis, and various regions of the brain, liver and white adipose issue. By contrast, ERβ is expressed in prostate (epithelium), colon, ovary (granulosa cells), bone marrow, vascular endothelium, salivary gland and certain regions of the brain. In some tissues, both ERs are expressed albeit in different cell types. For example, in human testis, ERα is present in spermatogonia and Sertoli cells, and both ERs are present in other cells, such as Leydig cells and spermatocytes [8]. The two ER isotypes have different ligand binding and transcriptional activities but their affinity for E_2 and classical AE are similar. Indeed, the similar structure of their C-terminal ligand binding pocket has made development of specific ERβ ligands challenging. However, ERβ, unlike ERα, binds phytoestrogens with high affinity. Although the ligand-binding properties of ERα and ERβ overlap, studies with knockout mice revealed that these two ERs have distinct and unique roles in vivo [92]. ERβ inhibits human ERα-positive BC cell proliferation by repressing transcription of the *c-myc*, *cyclin* D_1 and *cyclin A* genes and increasing the expression of $p21^{Waf1/Cip1}$ and $p27^{kip1}$, leading to cell cycle arrest in the G_2 phase [15]. ERβ is also able to inhibit the proliferation of ERα-negative BC cells and it decreases their invasiveness capacity [93]. The reported inhibition of tumor growth by ERβ in various mouse models in which ERβ opposes the proliferative effects of ERα [16, 94] has led to the suggestion that ERβ acts as a tumor suppressor [95]. Consistent with this view, ERβ inhibits angiogenesis and tumor growth in a T47-D xenograft model [94], and the siRNA-mediated knockdown of ERβ increases the expression of genes relevant to tumor cell

proliferation such as the pro-apoptotic Bik [96]. ERβ expression is linked to less aggressive tumors in BC suggesting that its re-expression in ER-positive tumors could be beneficial. Indeed, ERβ seems to potentiate the antiproliferative activity and apoptotic effects of 4-OH-Tam in BC cells [96]. Thus, ERβ re-expression in ER-positive or negative tumors may be therapeutically useful by decreasing the survival of p53-defective cancer cells after DNA damage. There are therefore good reasons to conduct trials combining the re-expression of ERβ following chemotherapy.

ERβ itself may be involved in Tam-induced resistance because ERβ expression increases the sensitivity of BC cells by down-regulating the ErbB-2/ErbB-3/AKT signaling. Indeed, re-expression of ERβ in MCF-7 and T47-D BC cells ($ERα^+$ but $ERβ^-$) decreases the formation of the ErbB-2/ErbB-3 receptor dimers and down-regulates their active regulator AKT, resulting in an increase sensitivity to Tam [97].

Only a few ligands exists that show high affinity and a potency preference for ERβ over ERα and their anticancer activity is currently under investigations (**figure 3**). Among them, racemic DPN, has a higher affinity for ERβ [98] but still has activity at ERα. It is therefore not yet established if stimulation of the transcription activity of ERβ is of therapeutic relevance or if the capacity of ERβ to hetero-dimerize with ERα is sufficient in itself to enhance the beneficial effects observed against BC proliferation and survival.

V.B. Membrane receptors and adaptor proteins

V.B. 1. Src kinase

Deregulation of the non-receptor c-Src cytoplasmic TK has been associated with many tumors including BC tumors, particularly in case of acquired resistance to treatments with either HT or anti-growth factors. Src and ERα together with PI3K are associated in several types of epithelial BC cells, where they form a complex, involved in non-genomic

pathway of E_2 induced cell proliferation [99]. In some cases, resistance is accompanied by an invasive phenotype concomitant with an increase of Src kinase activity [100]. Src regulates the chemokine CXCL12/SDF-1, helping indolent BC cells to survive in the bone marrow. CXCL12/SDF-1 also upregulates AKT expression, thus increasing survival and resistance to TRAIL death signals [101]. The use of the Src/Abl kinase inhibitor AZD0530 (**figure 8**) was shown to synergize with Tam [102] or gefitinib ("Iressa", an EGFR inhibitor) for suppressing the invasive phenotype at least in vitro [103]. The development of BEZ2235 (a dual nanomolar inhibitor of both PI3K and mTOR), is very promising for a new therapeutic approach [104]. Altogether, these findings suggest that inhibiting Src activity is a potentially useful therapeutic strategy, which most likely operates via preventing dormant cells from becoming a source of future metastasis in the bone marrow. Due to the crosstalk between Src and methylated ERα [6], it is likely that combining Src kinase inhibitors with PRMT1 inhibitors may improve reduction of BC cells invasion and metastasis. Src is constitutively activated in Trastuzumab-resistant BC cells; its targeting with specific inhibitors such as Saracitinib, re-sensitizes resistant BC tumors in xenografts to Trastuzumab [105]. This observation favors the notion of the combination of Src inhibitors with Erb-B2 targeted therapy would be beneficial.

V.B.2. The PI3 kinase/AKT pathway

The PI3K/ protein kinase B (AKT) pathway is a key regulator of cell proliferation and survival since PI3K-produced phospholipids favor the membrane recruitment of AKT which is itself further phosphorylated (activated) by either the 3-phospho-inositide-dependent protein kinase 1 (PDK1) or by the Ric/TOR complex. Such a cascade of events is critical for cell cycle progression and suppression of apoptosis [50]. Importantly, ERα binds in an

estrogen-dependent manner to the p85α regulatory subunit of PI3K, leading to the activation of AKT and endothelial nitric oxide synthase (eNOS) [23]. This provides an explanation for the cardiovascular protective effects of oestrogen. BC resistance to endocrine therapy can be associated with an invasive phenotype concomitantly with an increase of Src kinase activation and that of the mTOR intracellular signaling pathway [100]. Thus, targeting the Pi3K/AKT signaling must be considered as a prime strategy in cancer treatment particularly in BC where there are evident connections with membrane ERα. Many signals emanating from the membrane, including E_2 binding to either GPER or membrane incorporated ERα leads to phosphorylation of AKT after PI3K activation. As a consequence, cells are driven to progress in cell cycle and to survive (**figure 2**). In early studies, the mTOR inhibitor everolimus (**figure 8**) added to endocrine therapy showed antitumor activity. Everolimus combined with an AI improved progression-free survival in patients with hormone-receptor-positive advanced BC previously treated with non steroidal AIs. Furthermore, expression of ERβ in ERα-positive BC cells, such as MCF-7 and T47-D, results in a decrease of AKT signaling and down-regulation of HER2/HER3 dimers, concomitantly with a decrease of the natural inhibitor of AKT, PTEN [97]. These findings provide a strong support for a major relationship between several partners involved in resistance to AEs. They argue for initiatives to develop re-expression of ERβ in BC cells in order to improve BC cells sensitivity to AE and/or AIs.

V.B.3 Chemokine receptors

Many solid tumors including BC express high levels of various chemokine receptors (review in [106]). In addition, many chemokines are produced in larger amounts by epithelial cancer cells than normal epithelial cells and also by the tumor microenvironment, resulting in enhanced tumor cell proliferation, migration, angiogenesis and then bone metastasis.

The production of a number of chemokines or their receptors in BC can be linked to ER pathway. CXCL8 is secreted by BC cells and its titer inversely correlates to ER levels [106]. Similar findings have been reported for several other chemokines including CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CCL2 and CCL4 in BC patients [107, 108]. One must note that, the weak expression of chemokines such as CXCL8 in ER-positive BC could be the result of histone deacetylase inhibition in such cells [109].

The activation of the CXCR4/CXCL12-SDF-1 (Stromal cell-Derived Factor-1) pathway (**figure 2**) has also been implicated in acquired Tam resistance. In ER-positive BC cells, the chemokine CXCL12 and one of its receptor CXCR4 are induced by estrogens [110]. This could explain the positive correlation between CXCL12 and ER status in BC patients [111]. However, the regulation of CXCR4 by E_2 seems to be more controversial because another study did not find induction of CXCR4 by E_2 in wild-type MCF-7 cells, but observed E_2 induction in MCF-7 over-expressing Erb-B2 [112]. Significantly, CXCL12 and CXCR4 favor the hormone-independent growth of BC cells both in vitro and in vivo [110, 113]. Studies in vivo showed that CXCL12 can at least partially alleviate the anti-proliferative action of Faslodex implicating CXCL12 in hormone resistance [113]. E2-induced transcriptional activation of the *SDF1* gene (and possibly other ER-regulated genes) occurs through both ERs isoforms. In turn, SDF1 interaction with its CXCR4 receptor may induce a "feed forward" loop, leading to phosphorylation of both ERs through Erk activation, a mechanism which could explain BC cell growth and Tam resistance [114]. Therefore, targeting CXCR4 (through the inhibitor AMD3100, **figure 6**) and/or SDF1 could have a potential therapeutic usefulness.

V.B.4. The IGF axis

As described above, ligand activation of the IGF-1R and its downstream pathways (PI3K/AKT/mTOR and Ras/Raf/MEK/ERK) stimulates tumor proliferation, survival,

transformation, metastasis and angiogenesis [115] (**figure 2**). In ER-positive BC cells, activation of IGF-1R can negatively affect the efficacy of both AEs and chemotherapy. Estrogens reinforce the responsiveness of BC cells to IGF by inducing the expression of IGF-1R and IRS-1; in turn, IGF/IGF-1R signaling can activate the Erk1/2 kinases which can specifically phosphorylate ERα on ser418 and then activate ER-mediated transcription [116]. This constitutes a therapeutic potential in targeting the IGF-axis in BC. Indeed, inhibition of IGF-1R signaling is synergistic with endocrine therapy in preclinical models of ER-positive breast cancer. There have been many trials recently investigating IGF-1R as a possible cancer target. Major efforts have focused on the use of monoclonal antibodies against IGF-1R such as AMG-479 which blocks IGF-1 ligand-mediated activation and small TK inhibitors directed against the IGF-1R TK domain [117, 118]. Several chemical molecules are currently under intense investigation and in different experimental phases [119]. Available data suggest that this class of compounds is well tolerated with mild to moderate side effects, when used alone or in combination with other therapeutic agents. In a recent work [120], it has been demonstrated that E_2 and IGF-1 down-regulate critical repressors of BC growth (like the key suppressor of tumorigenesis B-cell linker or *BLNK*) by independent mechanisms. This is of clinical significance because the restoration of BLNK expression may limit progression of the disease; this could be achieved by combining AE with anti-IGF-1 molecules. *In vivo*, the activity of IGF is regulated by its binding to IGF-binding proteins (IGFBP 1-6) which complex almost 99% of circulating IGF thus serving as a reservoir for IGF. The development of a strategy consisting of maintaining this reservoir capacity to prevent the release of IGF and its subsequent activation of the IGF-1R is a novel possible approach to circumvent the detrimental effects of the IGF pathway on BC progression.

V.C HSP90

Following their synthesis in the ribosomes, all steroid receptors are associated in a multiprotein chaperone complex organized around Hsp90 [7] which helps to fold client proteins. This multistep folding process requires ATP binding to Hsp90 and other co-chaperones [121, 122]. HSP90 is necessary for ER and other NRs to display high affinity ligand binding and more generally for the full expression of the biological capacities of client proteins. HSP90 is a major player in the degradation through the ubiquitin-proteasome pathway of not only NRs but also many oncogenic signaling proteins including ErbB2, c-Myc, AKT, Raf-1 and also mutated p53 (review in [123]). Many HSP90 inhibitors blocking the protein in an ADP-binding form or the binding of ATP have been developed. These inhibitors disrupt client protein function and/or their degradation process and lead to apoptosis. Some of these inhibitors, notably geldanamycin (**figure 9**) and several coumarin derivatives [124-126], are potential anticancer therapeutic agents due to their capacity to induce apoptosis in a large variety of cancer cells. However, the multitude of targets in all cells renders these molecules extremely toxic and their clinical use has not to date been authorized. However, their incorporation in nanodevices targeting BC cells appears promising in preclinical models (our unpublished work).

VI. Conclusions and future directions

Hormonal treatment of BC is the first real example of successful targeted therapy. The development of AE and of new AIs has considerably enhanced the efficacy of the treatments but long term post-treatment resistance often develops. Deciphering the mechanisms underlying this resistance has identified new ways to reduce re-start of cell proliferation and survival. This is particularly true in case of targets like HSP90 and HDACs for which a number of new inhibitors has been synthesized. The use of new humanized

antibodies, other than herceptin, and targeting growth factor receptors are also promising. Several targets identified are of prime importance but are currently not accessible in vivo, because appropriate chemical inhibitors are not available (**Table 1**). Possibly, the targets involved in enhancement of tumor progression could be manipulated through the use of silencing RNAs or dominant negative constructs, but delivering such agents to cancerous cells remains a major challenge. This is particularly true in the case of miRNAs. MiRNAS are a class of naturally occurring, small (19-25 nucleotides) non-coding RNA molecules. They interact with mRNAs in their 3'-untranslated region and block mRNA translation or target the transcripts to degradation. Several miRNAs have been found in BC cells and some have been shown to be down-regulated by E_2, concomitantly with the enhanced expression of Bcl-2, cyclin D1 and survivin ([127] and references herein). Such miRNAs may also be considered as potential targets, although their way of administration is also challenging. Similar issues remain for targets whose expression needs to be increased, as for example, tumor suppressor genes. The types of biological molecules, required for this goal (plasmids, oligo-nucleotides), are fragile and need to be protected against degradation when injected in the body. They must also travel to reach a concentration sufficient in the tumor cells for obtaining a biological effect. Current progress justifies the development of appropriate methodology for the delivery of such molecules and this has indeed been achieved with nanocarriers [128]. More than 150 molecules are currently the subject of work on encapsulation in stable and non toxic formulations. Immunotargeting of such nanocarriers based on recognizing an over-expressed marker of BC cells and carrying strong inhibitors of the cell cycle or inducers of apoptosis are amongst the most promising strategies. For example, Erb-B2 is over-expressed in a number of BC tumors, particularly in those not responding to classical HT, accordingly Trastuzumab has been used in the fabrication of

Dacinostat-containing devices; these immunoliposomes substantially enhance programmed-cell death in BT474 BC xenografts [129]. Trastuzumab has also been conjugated (Trastuzumab-emtansine) to DM1, an inhibitor of tubulin polymerisation and clinical trials showed that this agent is effective in patients with metastatic triple negative BC [130]. Reaching metastasis remains a major obstacle in cancer therapy and immune-nanocarriers and/or antibody-conjugated chemicals seem promising tools for this goal.

Combinations of several molecules, free (like the combination Vorinostat-Tam in patient with hormone-resistant BC [131] or that of Tam with a Src inhibitor [132]) or encapsulated in stealth or tumor recognizing nanosystems, are in clinical trials, but the doses and sequence of administrations still need to be defined, because some combinations are incompatible when these conditions are not precisely optimized. This is particularly true in case of HDACis injected in combinations with Hsp90 inhibitors (our unpublished results). We think that the development of combinations associating tumor-piloted nanosystems carrying anticancer agents should be undertaken to circumvent hormone-resistance in BC.

Many combinations of conventional therapies are actually in various phases of clinical trials and more recent new treatment strategies have focused on epigenetic alterations. Histone acetylation but also DNA methylation are among the most common types of epigenetic modifications. Unlike gene mutations, these alterations are reversible, making them promising alternative targets in BC therapy. Similarly to HDAC inhibitors (see **figure 7**), DNA methylation inhibitors such as azacytidine and 5-aza-2'-deoxycitidine and pargyline have been approved by the FDA. They are known to slow the growth of MCF-7 and ZR 75.1 tumors in nude mice and to induce several prometastatic genes such as *UPA*, *CXCR4* and *TGFβ* by demethylation of their promoter [133]. In association with HDAC inhibitors, DNA

methylation inhibitors are also known to reactivate the ERα silenced gene in ER-negative MDA-MB-231 BC cells [60]. ERα is also found methylated at lysine 302 (K302) in MCF-7 cells by SET7 [134] a histone methyltransferase linked to p53 activation through interaction with the HDAC sirtuin1 [135] and methylated ERα is suggested to enhanced ER-transcription; thus inhibiting SET7 with methyl transferase inhibitors could be of therapeutical activity, and their incorporation in tumor targeted nanodevices could be useful to avoid unwanted side effects. Moreover, the recent discovery coupling LSD1/KDM1 to ERα and the positive regulation of the Erb-B2-aromatase pathway by the PELP1-LSD1/KDM1 signaling [79] have implicated LSD1 in hormone resistance. Inhibiting LSD1 as well as other methyl transferases could have a potential therapeutic strategy by impeding aromatase production and arresting BC growth ([80] and ref herein).

The development of gene strategies is also promising for BC treatment, as both positive re-activation of tumor suppressors, such as ERβ, LKB1 or wild-type p53, and also stopping expression of genes involved in tumor growth can be considered. This could be accomplished by the use of shRNA or siRNA to silence, for example AKT, AIB-1, Bcl-2, or VEGF. This approach was successfully used in BC MCF-7 cells models inoculated with PELP1-siRNA-loaded liposomes, resulting in slowing down tumor progression in xenografts [79]. Indeed, many trials are underway to study the use of antibodies targeting growth factor receptors and various inhibitors (TK, or HDAC, or others). However we believe that effective treatments are more likely to emerge from the development of targeted chemical molecules, whether encapsulated in nanocarriers or linked to antibodies against proteins over-expressed by tumors for specific delivery to the tumor sites.

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Legends of figures:

Figure 1: Classical mechanisms of ligand and phosphorylation-induced transcription in breast cancer cells and antiestrogen activities.

Estrogens like estradiol (E_2) and SERMs (such as tamoxifen, Tam) bind to ER associated in the HSP90-containing multichaperone complex, leading to the release of HSP90, dimerisation of ER and binding to DNA sequences. According to the "canonical model", following E_2-binding, ER dimers bind to specific 13-bp DNA sequences in the estrogen receptor response element ERE (CGGGTCAnnnTGACCTG) characteristic of specific E2-regulated genes such as *pS2*. DNA-bound ER-agonist complexes recruit several coactivators some with histone acetyl transferase (HAT) activities that allow repositioning of nucleosome and chromatin opening, and interaction with the general transcription machinery organized around RNA polymerase II; transcription subsequently begins. Note that other sequences recognized by intermediate transcription factors (such as AP-1 and SP-1) could serve as ER binding sites [136]. DNA-bound SERM-ER complexes (such as Tam-ER) recruit corepressors and histone deacetylases (HDACs) promoting re-compaction of chromatin and arrest of transcription. However, corepressors may be recruited also by ER-agonist complexes. This is particularly the case of

SMRT [137], demonstrating that the quality of comodulators which contribute to enhancement of E_2-induced transcription depends of the cellular and promoter context. The binding of pure anti-estrogens (such as Fulvestrant and RU) leads to relocalisation of ER in the endoplasmic reticulum where ER is ubiquitinated and rapidly degraded by the proteasome.

Figure 2: membrane emanating signals modifying estradiol responses.

E_2 may bind to ER localized at the membrane in interaction with Src and PI3K which are thereby activated and in turn activate mTOR through PI3K-mediated AKT phosphorylation (a). In addition, activated AKT may phosphorylate both E_2-free and -bound ER and enhance classical genomic transcription. These effects enhance transcription of genes involved in cell cycle progression and survival. The E_2-activated GPER can trigger the PI3K/AKT/mTOR pathway (b) which is also activated through the enhancement of TK activity of homo- and heterodimers of growth factor receptors (b'). Crosstalk has also been established between GPER and EGFR (c) which enhances the activation of the MAPK pathway. The IGF axis (d) participates in the activation of cell proliferation largely in case of resistance to treatments, and ERα is specifically activated by IGF-1R-induced phosphorylation (see text for details). The *SDF1* gene is activated by E_2 and the excreted SDF1 protein activates the chemokine receptor CXCR4 (e), which can also be a consequence of tamoxifen (Tam) binding to ERα. As a consequence, the MAPK pathway is activated and ER-mediated transcription can be enhanced in the absence of E_2. The inhibitions of some of the targets of ER by chemical molecules currently in clinical trials (red labels) and antibodies (blue arrows), involved in these strongly interacted pathways are indicated. LY = LY294002.

Figure 3: Molecular structure of NR, GPER ligands, and aromatase inhibitors.

17β-estradiol: (17β)-estra-1,3,5(10)-triene-3,17β-diol; **tamoxifen**: (*Z*)-2-[4-(1,2-diphenylbut-1-enyl)phenoxy]-*N*,*N*-dimethylethanamine; raloxifene: [6-hydroxy-2-(4-hydroxyphenyl)-benzothiophen-3-yl]- [4-[2-(1-piperidyl)ethoxy]phenyl] –methanone; **Fulvestrant**: (7α,17β)-7-{9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl}estra-1,3,5(10)-triene-3,17-diol**; RU 58668:** (7α,17β)-7-{9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl}estra-1,3,5(10)-triene-3,17-diol**;** **genistein**: 4',5,7-trihydroxyisoflavone, 5,7-dihydroxy-3-(4-hydroxyphényl)-4H-1-benzopyran-4-one; **DPN:** 2,3-*bis*(4-Hydroxyphenyl)-propionitrile; **PHTPP**: 2,3-*bis*(4-Hydroxyphenyl)-propionitrile, ERβ antagonist (anti); **aminoglutetimide**: (*RS*)-3-(4-aminophenyl)-3-ethyl-piperidine-2,6-dione; **Exemestane**: 6-Methylideneandrosta-1,4-diene-3,17-dione; **Anastrozole**: 2,2'-[5-(1H-1,2,4-triazol-1-ylméthyl)-1,3-phénylène]bis(2-methylpropanenitrile); **Letrozole**: 4,4'-((1H-1,2,4-triazol-1-yl)methylene)dibenzonitrile; **MIBE**: ethyl 3-[5-(2-ethoxycarbonyl-1-methylvinyloxy)-1-methyl-1H-indol-3-yl]but-2-enoate.

Figure 4: IGF-1R tyrosine kinase inhibitors

Figures 5: Erb-B2/HER tyrosine kinase inhibitors

Figure 6: Structures of Gossypol (SRC 1-3 inhibitor), AMI1 (PRMT1 inhibitor) and AMD3100-Plerixafor (CXCR4 inhibitor)

Figure 7: Structures of HDAC inhibitors

Figure 8: Structures of some Src (a, b), PI3K/AKT (c, d, e) and mTOR (f) inhibitors.

Figure 9: Hsp90 inhibitors

Legend of table

Table 1: targets impacting ER-mediated functions in breast cancer cells.

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Targets	activity	Molecules/ Gene manipulation
ERβ	ERα dominant negative	No specific ligand known to arrest BC cell growth. Over-expression by gene transfer
HDAC	Histone deacetylase	TSA ; M344 ; BML210 ; Panabinostat ; Mocetinostat ; Docinostat ; Entinostat ; Romidepsin
SRC1-3	ERα Co-act	Gossypol
E6AP	ERα Co-act, E3-ubiquitin ligase	No drug known. Over-expression by gene transfer
PRMT1	Arginine methyl transferase	siRNA transfer
LSD1; SET-7	lysine methyl transferase	pargyline (LSD1); siRNA transfer
PELP1	ERα Co-act	No drug known; siRNA transfer
LKB1	ERα Co-act; ser/Thr kinase	Metformin, over-expression
HOXB7	Transcription factor	No drug known ; siRNA transfer
TLE1	Modulator of ERα-mediated transcription	No drug known; siRNA transfer
PAX2	Mediator of ERα-mediated repression of Erb-B2 by Tam	No drug known; siRNA transfer
FOXA1	Regulator of ERα-chromatin interaction	No drug known; siRNA transfer
PI3K/AKT	Ser/thr kinase,	LY2940002, BEZ235, CUDC-907
Src	Tyr kinase	Dasatinib, Bosotunib, Saracatinib, BEZ235
mTOR	Ser/thr kinase,	Everolimus, Temsirolimus

IGF-1R	Tyr kinase	OSI-906; ADW742; BMS554417; PQIP; BMS754807
GPER	Membrane E2-binding receptor	MIBE, G15, G36
HER	Growth factor membrane receptor TK	Lapatinib, Gefitinib, Erlotinib
Hsp90	Molecular chaperone	17AAG; Radicicol; Ku 398; 4TCNA; 6BrCaQ
CXCR4/CXCL12 (SDF1)	ERα-enhanced mitogenic pathway	AMD3100; CXCR4 dominant negative over-expression or siRNA transfer

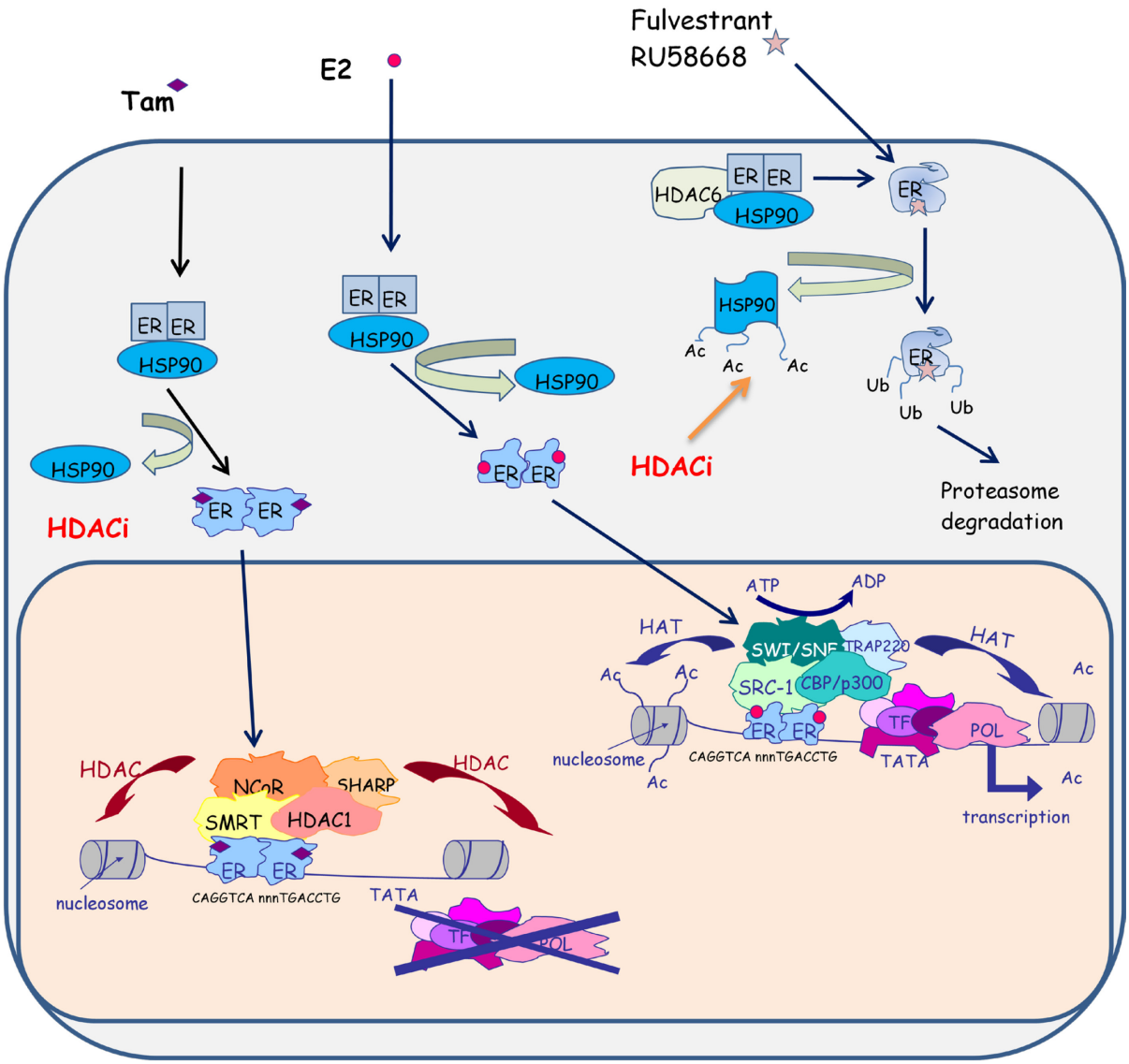

Fulvestrant
RU58668
E2
Tam
ER ER
HSP90
HDAC6
ER
Ac
Ub
HDACi
Proteasome
degradation
ATP
ADP
HAT
SWI/SNF
TRAP220
SRC-1
CBP/p300
TF
POL
TATA
nucleosome
CAGGTCA nnnTGACCTG
transcription
HDAC
NCoR
SHARP
SMRT
HDAC1

Figure 1

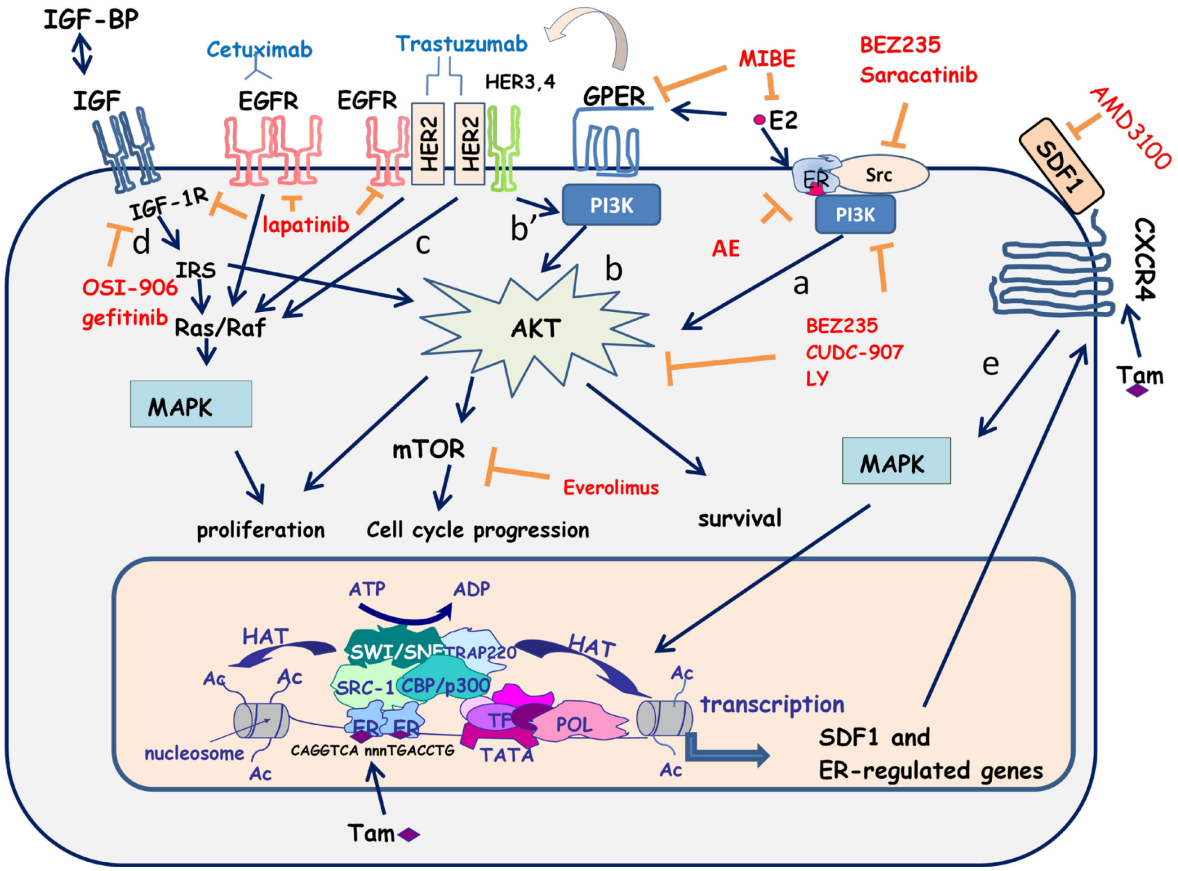

IGF-BP
IGF
Cetuximab
EGFR
EGFR
Trastuzumab
HER2
HER2
HER3,4
GPER
MIBE
E2
BEZ235
Saracatinib
AMD3100
SDF1
CXCR4
Tam
ER
Src
PI3K
PI3K
IGF-1R
lapatinib
AE
d
c
b'
b
a
e
IRS
OSI-906
gefitinib
Ras/Raf
AKT
BEZ235
CUDC-907
LY
MAPK
MAPK
mTOR
Everolimus
proliferation
Cell cycle progression
survival
ATP
ADP
HAT
HAT
SWI/SNF
TRAP220
SRC-1
CBP/p300
Ac
Ac
Ac
Ac
Ac
ER
ER
TF
POL
TATA
nucleosome
CAGGTCA nnnTGACCTG
transcription
SDF1 and
ER-regulated genes
Tam

Figure 2

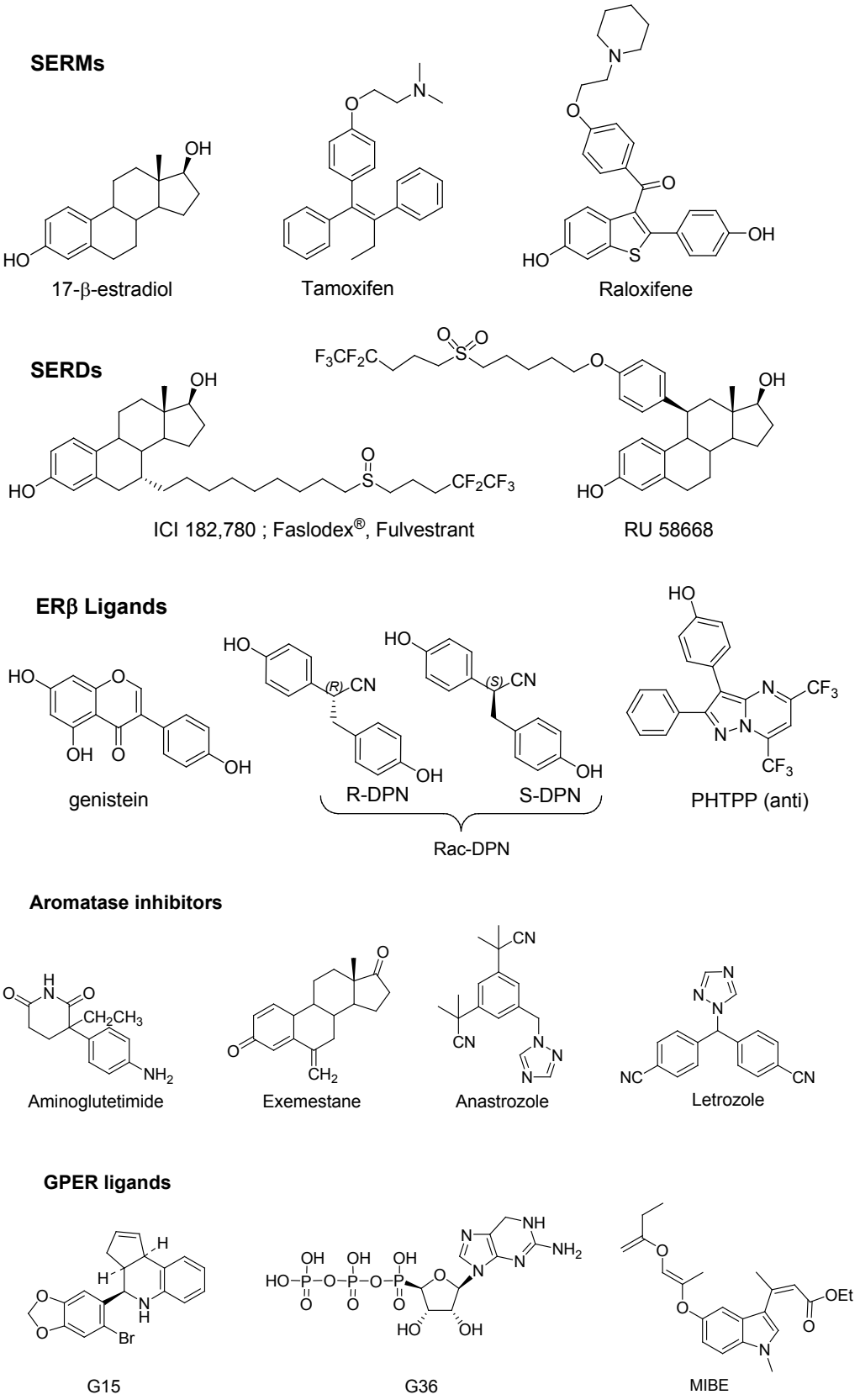

SERMs
17-β-estradiol
Tamoxifen
Raloxifene
SERDs
ICI 182,780 ; Faslodex®, Fulvestrant
RU 58668
ERβ Ligands
genistein
R-DPN
S-DPN
Rac-DPN
PHTPP (anti)
Aromatase inhibitors
Aminoglutetimide
Exemestane
Anastrozole
Letrozole
GPER ligands
G15
G36
MIBE

Figure 3

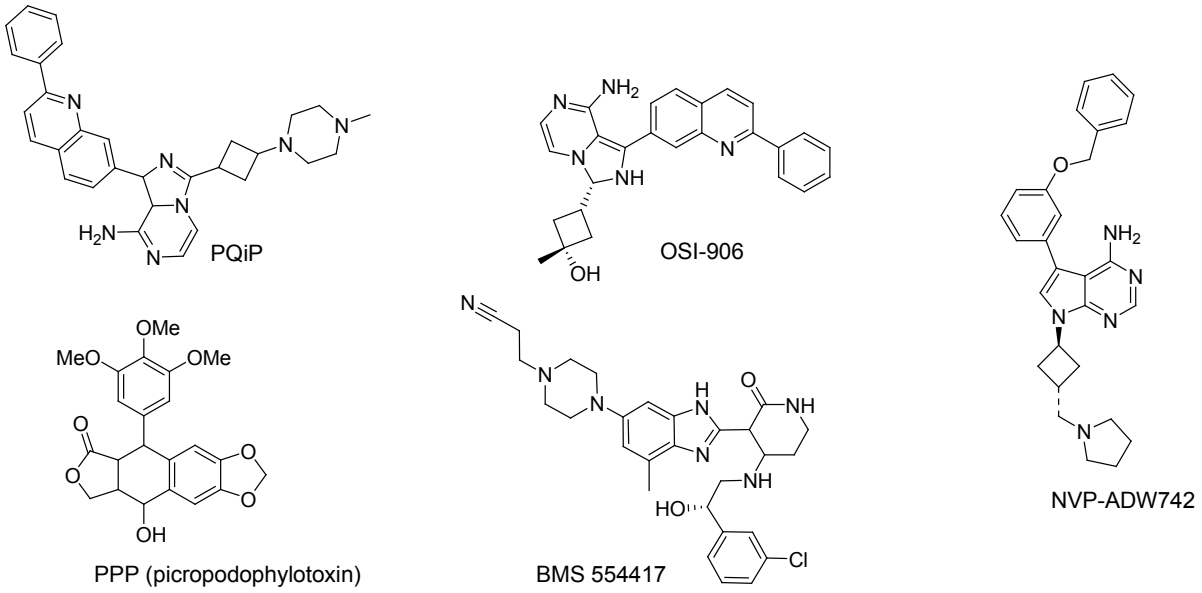

PQiP
OSI-906
NVP-ADW742
PPP (picropodophylotoxin)
BMS 554417

Figure 4

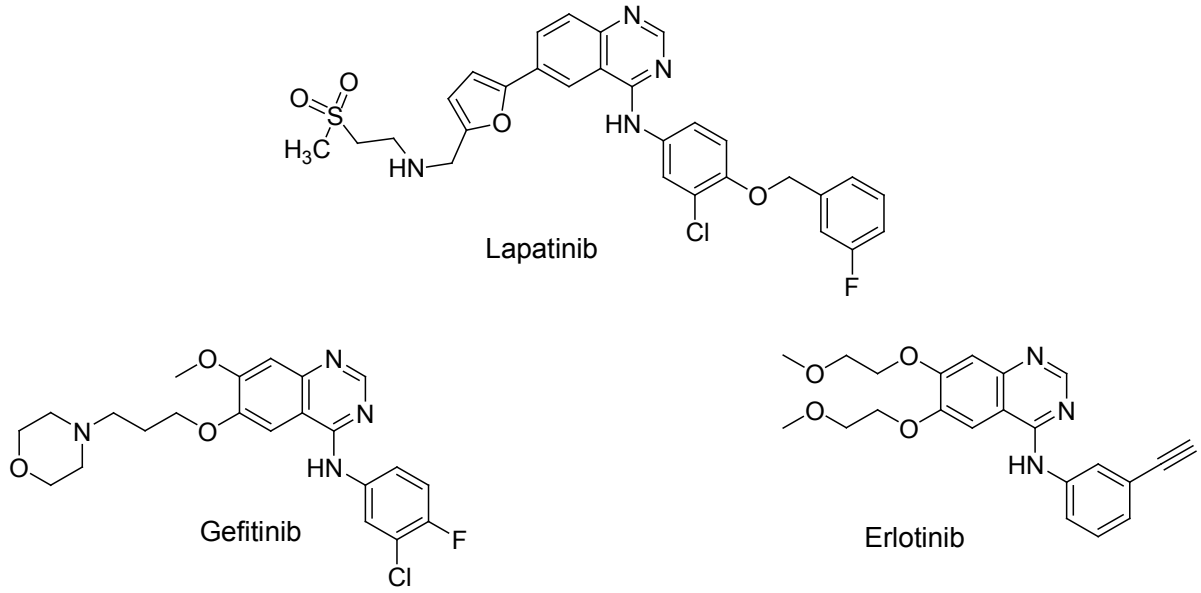


Figure 5

Figure 6

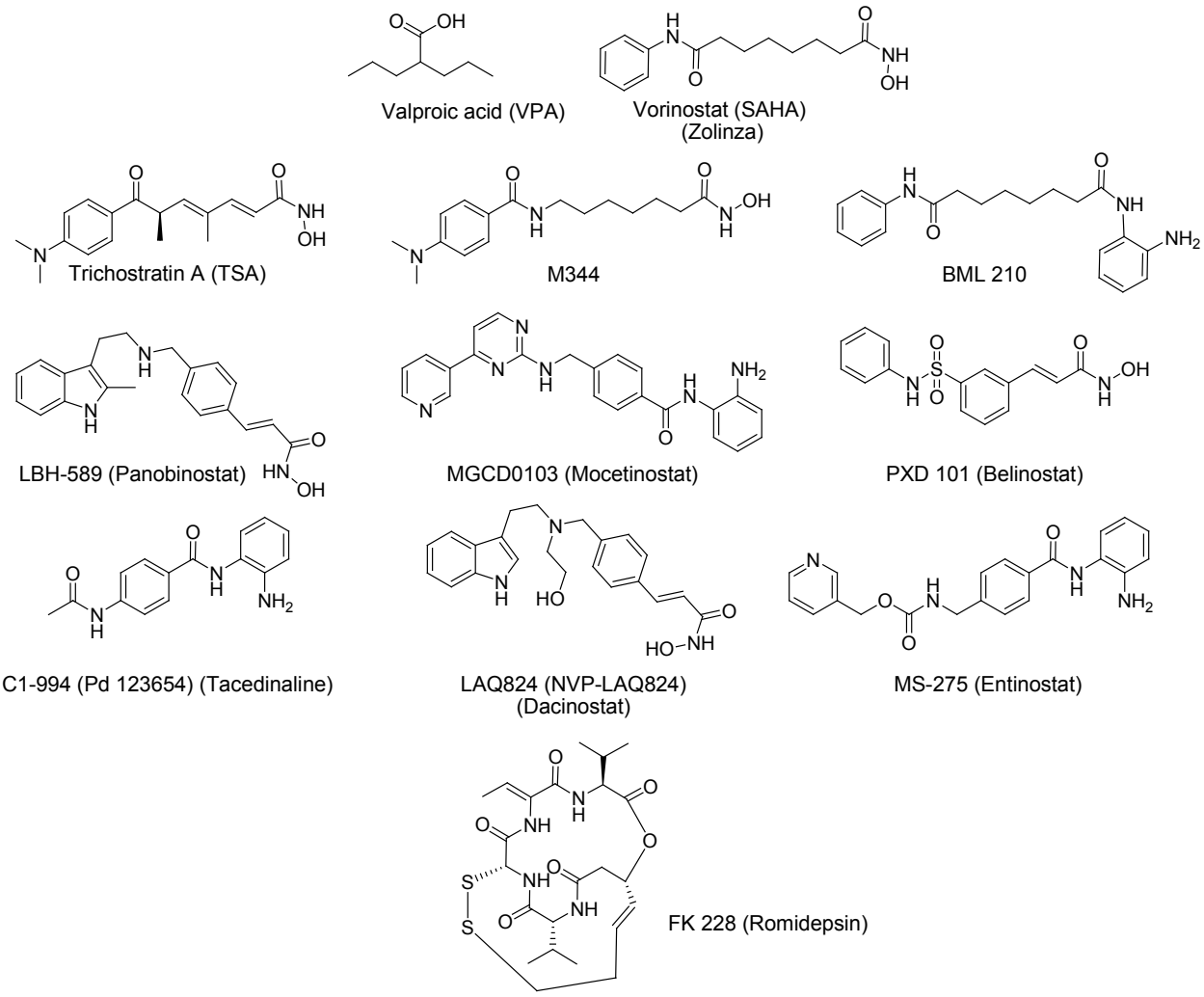
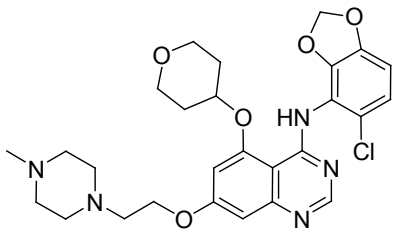
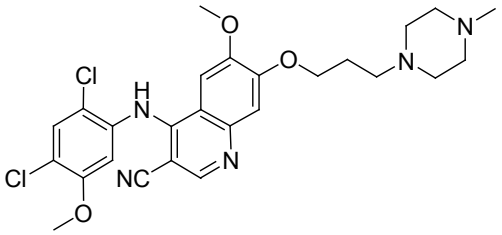

Valproic acid (VPA)
Vorinostat (SAHA)
(Zolinza)
Trichostratin A (TSA)
M344
BML 210
LBH-589 (Panobinostat)
MGCD0103 (Mocetinostat)
PXD 101 (Belinostat)
C1-994 (Pd 123654) (Tacedinaline)
LAQ824 (NVP-LAQ824)
(Dacinostat)
MS-275 (Entinostat)
FK 228 (Romidepsin)

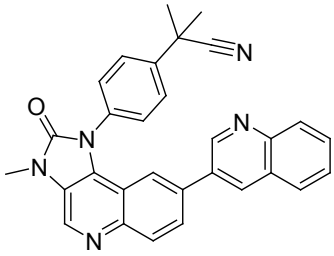
Figure 7



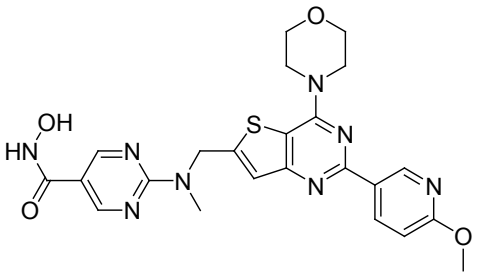
a) AZD 0530- Saracatinib
(Src/Bcr-Abl)



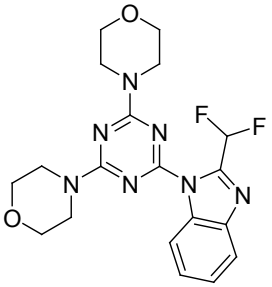
b) Bosotunib
(Src/Abl)



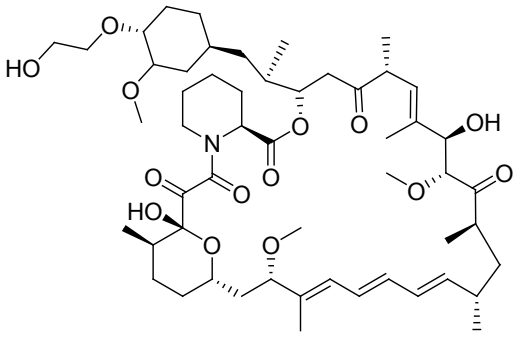
c) NVP - BEZ 235
(PI3K - mTOR)



d) CUDC907
(PI3K and HDAC)



e) ZSTK474
(PI3K)



f) Everolimus
(mTOR)

Figure 8

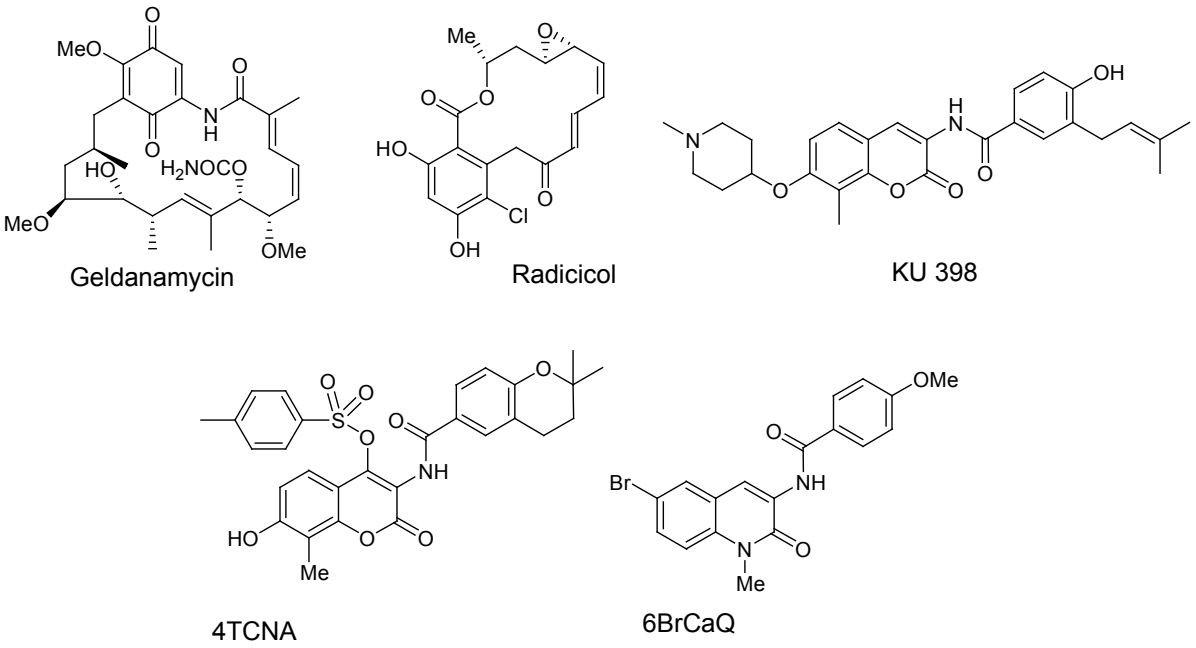
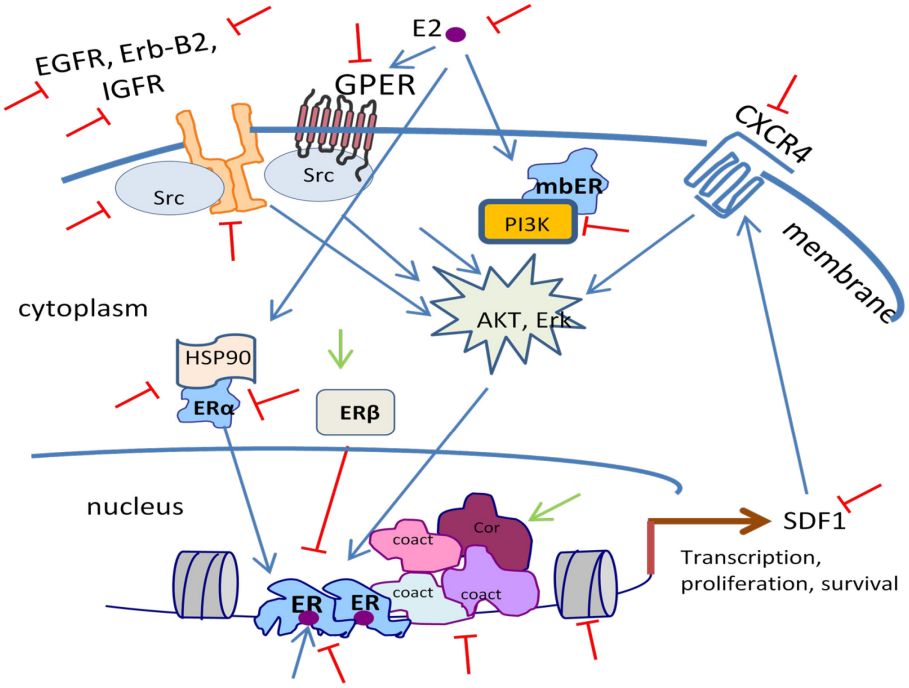

MeO
O
N
H
HO
H2NOCO
MeO
OMe
Geldanamycin
Me
O
HO
Cl
OH
Radicicol
OH
H
N
N
O
KU 398
O
S
NH
HO
Me
4TCNA
OMe
Br
NH
N
Me
6BrCaQ

Figure 9



Schematic representation of some cytoplasmic, membrane and nuclear estrogen receptor (ERα) targets : estradiol (E2) activation of ERα signaling is indicated by blue arrows; activation/enhancement of targetable proteins is indicated bygreen arrows, and inhibition with with red T symbol.

Graphical artwork