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Promise and failure of targeted therapy in breast cancer

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TABLE OF CONTENTS

1. ABSTRACT

The current molecular targets in breast cancer (BC) clinical trials were identified before the advent of the genomic era and their relevance was confirmed and validated by the introduction of gene profiling. Pioneering molecular analyses and repeated data validations on different gene platforms have thus far served to define 5 subtypes of BC based on their gene signature: luminal A, luminal B, normal-like, HER2-positive, and basal. Luminal A and B tumors are estrogen receptor (ER)-positive, while basal-like are mostly negative for ER, progesterone receptor, and HER2, i.e., triple-negative. Normal-like tumors resemble normal breast tissue and the HER2 subtype is characterized by HER2 overexpression. Here, we summarize current targeted therapeutic options for the luminal, HER2-positive, and basal-like BC subtypes with respect to results observed in clinical trials as a step toward optimizing their appropriate application in the different clinical settings. We give particular consideration to the ER- and HER2-targeted therapies approved for clinical practice with respect to their merits and shortcomings in early and advanced disease, and mention the therapeutic options currently available and potentially promising for the basal-like subtype.

2. INTRODUCTION

The mechanisms underlying the onset of BC are complex and vary among individual tumors (1; 2). These mechanisms include genetic and epigenetic alterations and resulting changes in the activity of signaling pathways. Mutations or epigenetic functional inactivation of tumor suppressor genes may contribute to the early development of some tumors, and alterations in proto-oncogenes may also be involved (3). Because altered patterns of gene expression are associated with corresponding variations in growth rates and cellular composition (4), analyses of these gene expression patterns can help to define tumor subtypes. Indeed, expression array analyses have served to identify different BC molecular subtypes with distinct clinical behaviors: basal-like, HER2, luminal A and B, and normal breast-like. Basal-like tumors are the most undifferentiated BC malignancies, characterized by the absence of expression of hormone receptors or HER2 and by a high expression of genes typical of the basal epithelial cell layer. HER2 tumors overexpress HER2 and multiple genes from the 17q11 amplicon. Although both luminal A and luminal B tumor subtypes express luminal cell markers and are mostly ER-positive (some luminal B tumors do not express ER), they differentially express certain proteins and differ in clinical outcomes (5).

Table 1. Relationship between molecular classification of BC and targeted therapeutics

Tumor subtype	**Targeted therapy**[1]	
	FDA/EMEA-approved	Under investigation
Luminal	TAM, AI	TAM + TKI
HER2	Trastuzumab, Lapatinib	TAM + Lapatinib, Pertuzumab+Trastuzumab, Pertuzumab+Lapatinib, Bevacizumab, Trastuzumab-DM1
Basal-like	Not available	Bevacizumab, Sunitinib, Cetuximab, PARP inhibitors

[1]The majority of these agents are administered in patients after, in combination with or sequential to chemo- or hormone therapy, as described in the text.

Two markers, the ER and HER2, are routinely used to select patients for hormonal treatment and anti-HER2 therapy, respectively; however, expression of these markers does not guarantee response, and their lack of expression is considered a potent argument to avoid specific therapy (1). In light of extensive laboratory data and clinicopathological correlations indicating that angiogenesis plays an essential role in BC development, invasion and metastasis, the anti-angiogenic agent bevacizumab directed to the vascular endothelial growth factor (VEGF) pathway has been approved for the treatment of metastatic BC, particularly in combination with chemotherapy. Systemic treatment for patients with HER2-negative disease is still limited to endocrine and cytotoxic therapies, and choices are even more limited for patients with triple-negative tumors, who currently do not benefit from targeted therapy. In these patients, different trials are ongoing to evaluate clinical benefits mediated by multi-targeted tyrosine kinase inhibitors (TKIs) with anti-angiogenic and anti-proliferative activities. However, despite all current advances, inherent and acquired tumor resistance remains a significant problem in ongoing research, and many efforts are dedicated to resolving this important issue. The common concept of resistance to therapy derives from clinical evidence that some tumors do not benefit from a treatment active on other similar tumors, suggesting that not only the biology of the tumor, but also the doses, time of delivery and combination of drugs determine response.

3. LUMINAL SUBTYPES

The estrogen receptor (ER) pathway is key for survival and progression in a significant proportion of BC, i.e. luminal subtypes, even further analyses indicated that luminal subtype tumors can also exhibit expression of growth factor receptors (e.g. HER family) and related genes. "Highly marked," indicating the predictive power of hormone receptor positivity for benefit from interventions that impair ER signaling, is characteristic of luminal BC. Targeted ER therapy for this BC subtype was first used over a century ago in the form of bilateral oophorectomy. It has evolved to ER modulators, such as tamoxifen and raloxifene, and aromatase inhibitors (anastrozolo/letrozolo) to become the most effective and, arguably, least toxic systemic therapy for luminal BC (Table 1).

3.1. Tamoxifen (TAM)

TAM, a non-steroidal agent, is an antagonist of the ER in breast tissue and has been the standard endocrine (anti-estrogen) therapy for hormone-positive early BC in post-menopausal women, although aromatase inhibitors (AIs) have also been proposed (6). In 1962, Dora Richardson first synthesized TAM, known then as TAM ICI-46,474, at ICI Pharmaceuticals (now AstraZeneca). The original trials of adjuvant versus control that began in the 1970s quickly showed that both local and distant recurrences could be delayed (7; 8). 1980 saw the publication of the first trial to show that TAM given in addition to chemotherapy improved survival of patients with early BC (9). In advanced disease, TAM is now only recognized as effective in ER-positive patients; note that the early trials did not select for ER-positive patients, and by the mid-1980s, the clinical trial picture was not showing a major advantage for TAM (10). Nevertheless, TAM had a relatively mild side-effect profile so that a number of large trials continued. It was not until 1998 that a meta-analysis by the Oxford-based Early Breast Cancer Trialists' Collaborative Group showed definitively that TAM saved lives in early BC (11); in 75 to 80% of patients with early ER-positive BC, treatment with TAM immediately and substantially reduces local, contralateral, and distant recurrence rates and reduces 15-year BC mortality (12).

TAM competitively binds to the ER on tumors and other tissue targets, producing a nuclear complex that decreases DNA synthesis, inhibits estrogen effects, and causes cells to remain in the G_0 and G_1 phases of the cell cycle. Because it prevents (pre)cancerous cells from dividing but does not cause cell death, TAM is cytostatic rather than cytotoxic. TAM itself is a prodrug, with relatively little affinity for its target protein, the ER. It is metabolized in the liver by the cytochrome P450 isoforms CYP2D6 and CYP3A4 into active metabolites, such as 4-hydroxytamoxifen and N-desmethyl-4-hydroxytamoxifen (endoxifen) (13), which have 30-100 times more affinity with the ER than TAM itself. These active metabolites compete with estrogen in the body for binding to the ER. In breast tissue, 4-hydroxytamoxifen acts as an ER antagonist so that transcription of estrogen-responsive genes is inhibited (14).

In contrast to estrogens, their antagonists induce a distinct receptor conformation leading to ER association with corepressor complexes, such as nuclear-receptor corepressor 1 (NCoR1) and NCoR2 (SMRT), rather than with coactivators, thereby shutting off gene transcription (Fig.1A and B) (15; 16). Interestingly, selective ER modulators (SERMs), including TAM and raloxifene, have a mixed agonist/antagonist activity and may either stimulate or antagonize ER function depending on the tissue, cell, and gene context (17).

Many coregulatory proteins may be present at rate-limiting levels in the nucleus, so that changes in their level of expression and/or activity can lead to alterations in

ER signaling. In particular, overexpression of coactivators and downregulation of corepressors can negate the inhibitory effects of endocrine therapy, especially in the case of SERMs (18-22).

Importantly, signaling from different growth factor receptor-dependent kinases activates various factors in the ER pathway, including ER itself, to potentiate ER genomic signaling activity on gene transcription (Fig.1 C) (23-25). Similarly, activation of the growth factor-dependent signaling of p42/44 MAPK (ERK 1/2) and phosphatidyl inositol 3-kinase (PI3K)/AKT leads to an increase in ER phosphorylation and ER AF-1 activity (26-28). This phosphorylation of ER and its coregulatory proteins by growth factor receptor-dependent kinases is an essential component of the ordinary regulation and function of genomic ER activity. However, in the presence of hyperactive receptor signaling, as often occurs in BC (e.g., HER2 overexpression), excessive phosphorylation of ER and its coregulators may severely weaken the inhibitory effects of various endocrine therapies and lead to endocrine resistance.

Estrogen, as well as some SERMs such as tamoxifen, exerts rapid stimulatory effects on a variety of signal transduction pathways and molecules. This activity is mediated, at least in part, by a small fraction of the traditional ER protein or perhaps by its closely related short-form splicing/translational variants (29-31) that are localized near or at the plasma membrane. Membrane ER may exist as a cytoplasmic pool tethered to the inner face of the plasma membrane bilayer through binding to membrane proteins of lipid rafts such as caveolin-1 (32; 33), flotillin-2 (34), or the caveolin-binding protein striatin (35), or possibly through association with other membrane receptors, e.g., insulin growth factor receptor (IGFR) (36), epidermal growth factor receptor (EGFR) (37; 38), or HER2 (34; 39), or with signaling adaptor molecules such as Shc (36). Recent laboratory data further suggest that the ER protein within isolated caveolar vesicles typically spreads throughout the cell membrane in a fashion similar to that of growth factor receptors (32; 40-42) and assembles as part of a large signalsome complex that includes receptor tyrosine kinases (RTKS), i.e., EGFR, IGFR, and HER2, as well as non-RTKs such as Src (43), and also G proteins (40; 44; 45). Studies in BC culture models have shown that the endogenous membrane ER can directly or indirectly activate EGFR, HER2, and IGFR1 (46). This process involves the sequential activation of Src (47), matrix metalloproteinases (MMPs) 2 and 9, and the release of the EGFR ligand heparin-binding EGF-like growth factor (HB-EGF), which, in turn, activates the EGFR downstream kinase cascades (Ras/Mek/MAPK and PI3K/AKT) (48; 49). These downstream activated kinases consequently phosphorylate and thereby activate ER and its coregulators, augmenting genomic activities of ER on gene transcription (26; 50; 51) (Figure 1D). Thus, the genomic and non-genomic mechanisms of action of ER do not appear to be mutually exclusive, but instead complementary to one another, and many interactions between these two signaling forms exist. These two ER activities also intimately interact at multiple levels with many cellular (growth factor-dependent and other) kinase networks to sustain bidirectional cross-talk that augments signaling of both ER and kinase-related pathways. The ER coactivator AIB1 is phosphorylated and activated by multiple kinases, including MAPKs and other cellular kinases (52-54).

Two independent recent retrospective studies demonstrate that tumors with high levels of both AIB1 and HER2 or HER3 are less responsive to TAM therapy, probably because of increased estrogen agonistic activity of TAM-bound ER (55; 56). Such findings support the hypothesis that increased signaling from the HER family (EGFR, HER2, HER3 and HER4) activates downstream kinases, which in turn activates ER and AIB1 to increase their transcriptional activity, including that in the presence of TAM. This mode of ER signaling might, therefore, be predominant in BC cells that express high levels of RTKs such as EGFR and HER2. Importantly, it has also been suggested that SERMs such as TAM may behave as estrogen agonists for these membrane effects of ER (Figure 1E) (50; 57).

Growing evidence indicates that HER2 activity underlies resistance to tamoxifen. Using the MCF7/HER2-18 cell line, which stably overexpresses endogenous AIB1 and exogenous HER2, Shou *et al.* (57) recently demonstrated that in a low-estrogen environment, TAM acts as a potent agonist on tumor growth. In MCF7/HER2-18 cells, both estrogen and TAM induces rapid (non-genomic) activation of EGFR/HER2 signaling, which leads to activation of both p42/44 MAPK and AKT signal transduction pathways. Short-term culture of these cells with TAM increased the expression of estrogen-regulated genes nearly as well as estradiol itself. This phenomenon is due to the ability of TAM-ER complexes to recruit coactivators such as AIB1 rather than corepressors to ER-targeted promoters in these HER2-overexpressing cells. Interestingly, all of these phenomena could be blocked by treatment with the selective EGFR TKI gefitinib, suggesting that EGFR/HER2 signaling is directly involved in the growth-promoting activity of TAM in HER2-overexpressing cells. These findings are in agreement with the clinical observations noted earlier indicating that tumors coexpressing HER2 and AIB1 have poor outcome when treated with TAM (55; 56).

Recent laboratory and clinical studies have shown that acquired resistance to TAM in tumors originally expressing low levels of EGFR and HER2 is also associated with increased EGFR/HER2 signaling, including HER2 gene amplification (58-62). As in the *de novo* experimental models of TAM resistance, the growth of these cells after acquiring resistance is significantly inhibited by treatment with the EGFR/HER2 inhibitor gefitinib or the monoclonal anti-HER2 antibody Trastuzumab (62; 63). Another RTK, the IGFR, has also been associated with TAM resistance. In fact, it was recently reported that IGF-II treatment activates both IGFR and EGFR/HER2 in TAM-resistant cells (64). Together, these findings suggest that enhanced growth factor signaling, which upregulates both the genomic and non-genomic activities of ER, is a key contributor to the

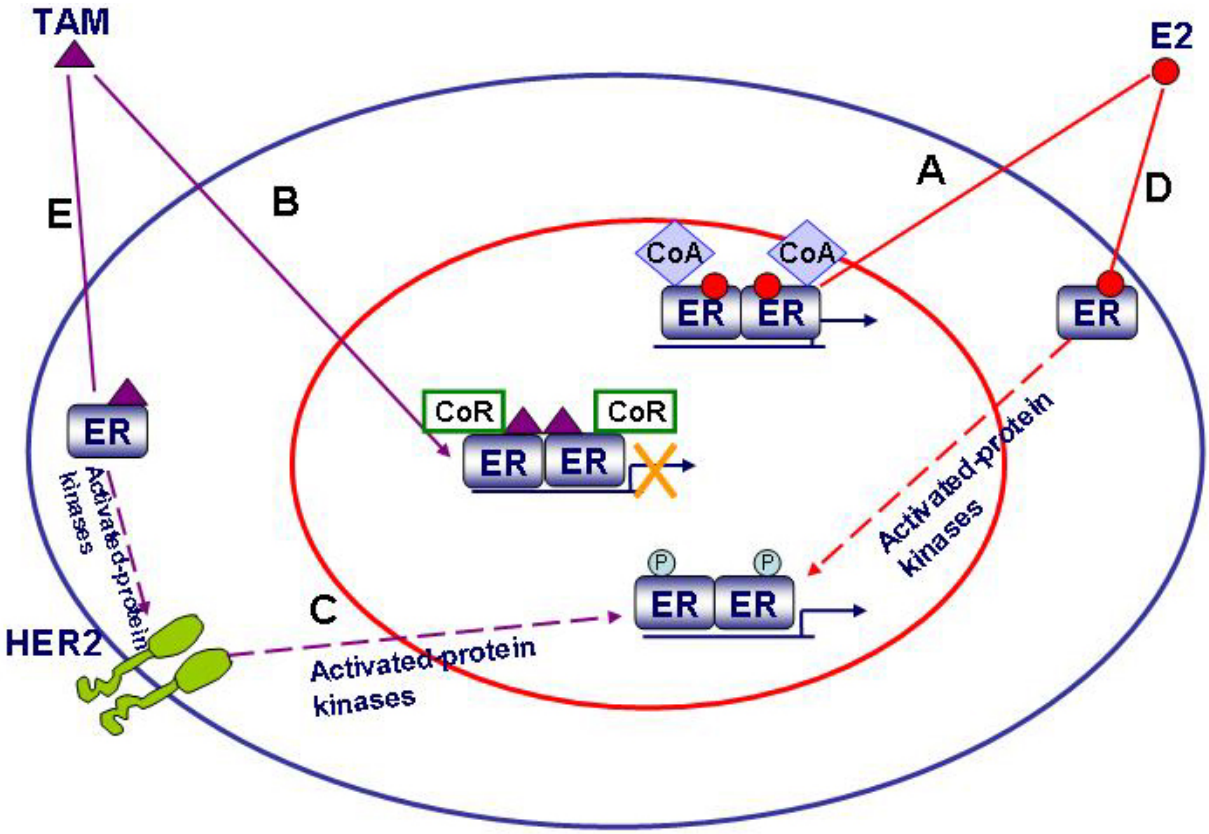

Figure 1. Tamoxifen activity. A: Ligand-bound estrogen receptor (ER) activates gene expression through binding to DNA response elements in complexes including co-activators (CoA); B: Tamoxifen induces a distinct receptor conformation leading to ER association with co-repressor (CoR) complexes; C: Signaling from growth factor receptor, e.g., HER2, activates various factors in the ER pathway, including ER itself, potentiating ER genomic signaling activity; D: ER can activate growth-factor signaling pathways with consequent phosphorylation of ER and its coregulators and augmenting genomic ER activity; E: TAM behaves as estrogen agonist, interacting with HER-dependent kinase networks to augment signaling of kinase-related pathways.

mechanism of acquired resistance to TAM. The convergence of many potential resistance genes on the ERK and PI3K pathways suggests that inhibitors of these pathways currently under development may be useful in this setting. Data from cell line models indicate that inhibiting Src118, BCAR1 (65), MEK–ERK (66), AKT (67) mTOR (68; 69) or NF-κB (70; 71) can restore or potentiate TAM sensitivity

A recently published meta-analysis examining the interaction between HER2 expression and response to endocrine treatment in metastatic disease clearly shows that HER2-positive BC is less responsive to endocrine treatment compared to HER2-negative (72).

EGFR generates similar, although not identical, downstream signals as HER2. In metastatic BC patients, EGFR overexpression is also predictive of a decreased benefit from TAM (73; 74). In a recent study (75), tumors with higher EGFR were less likely to respond to TAM, and these patients had a significantly shorter time to treatment failure. Even when ER and PgR levels were taken into consideration, EGFR remained predictive of a less sustained response, supporting the hypothesis that signaling from HER family members other than HER2 can also contribute to the development of TAM resistance. Indeed, a positive association between overexpression of HER family receptors (EGFR and/or HER2 and/or HER3) and TAM outcome has recently been shown in two additional independent datasets of patients treated with adjuvant TAM (76; 77). In the neoadjuvant setting, results confirm evidence for the role of HER2 and, to a lesser extent, of EGFR in TAM resistance (78; 79).

Recent provocative clinical studies (58) strongly suggest that acquired resistance to TAM may also be associated with an increase in HER2 expression and/or gene amplification, as shown in preclinical models. Acquired HER2 gene amplification during cancer progression after TAM adjuvant therapy has also recently been reported in patients' circulating tumor cells (59). Similarly, positive serum HER2 conversion, a circulating biomarker of HER2-positive BC progression (80), has also been shown in patients with advanced disease at the time of disease progression on endocrine therapy (76). These data suggest that acquired HER2 overexpression can occur during endocrine treatment, perhaps as an adaptive mechanism for tumor cell survival upon these therapies or

as a consequence of reversing ER-induced down-regulation of HER expression by endocrine therapy (81).

In vitro, the combination of TAM and the selective EGFR TKI gefitinib provided nearly complete inhibition of p42/44 MAPK and AKT phosphorylation, greater suppression of the cell-survival protein Bcl-2, and (63). For *de novo* TAM-resistant BC models involving cells stably transfected with HER2, endocrine therapy combined with different HER inhibitors is also more effective than the use of either therapy alone (57; 66; 82-85). *In vivo*, gefitinib in combination with TAM has been shown to completely overcome the agonist activity of TAM and significantly delay the growth of stably transfected HER2-positive MCF-7 xenografts (57). Similar beneficial effects were seen with gefitinib combined with estrogen deprivation (86). Finally, a recent study (82) demonstrated that in both MCF7/HER2-18 and BT474 ER-positive/HER2-positive xenograft tumors, a combination of several HER inhibitors designed to completely inhibit signaling from all HER dimer pairs, together with either TAM or estrogen deprivation (in the case of MCF7/HER2-18), is much more effective in inducing apoptosis and slowing proliferation than each individual drug.

Several phase II/III trials have been initiated with TKIs or monoclonal antibodies in combination with anti-estrogens (87-90). Some of these trials are in the second-line setting, including patients whose tumors were progressing on TAM and in whom the dual TKI lapatinib (anti-HER1 and anti-HER2) was used to determine whether clinical responses and TAM resistance reversal might occur. However, most of these studies are randomized phase II trials with only 100-200 patients. Moreover, the primary efficacy endpoint in some of these studies is objective response rate, whereas given the preclinical data, prolongation of time to progression (i.e., delaying resistance onset) might be a better endpoint for these trials.

Future and ongoing clinical trials will determine the true potential and applicability of this combined therapeutic approach. Further clinical trials are needed to evaluate various signaling elements from the multiple networks that cross-talk with and modulate ER activity as predictive markers for initial endocrine therapy. A molecular profile of these different components in a given patient's tumor immediately before treatment or at the time of disease progression after therapy might permit the individualization of both the initial type of endocrine therapy and the appropriate signaling inhibitor needed to block *de novo* or acquired resistance.

3.2. Aromatase Inhibitors (AIs)

AIs are a class of drugs used in the treatment of BC and ovarian cancer in post-menopausal women. In contrast to pre-menopausal women, in whom most of the estrogen is produced in the ovaries, post-menopausal women produce estrogen mosly in the adrenal gland from the conversion of androgens. AIs inhibit the action of the enzyme aromatase, which converts androgens into estrogens by a process called aromatization. Because estrogens stimulate breast tissue, decreasing their production is a way of suppressing recurrence of breast tumor tissue.

Initial studies of AIs in patients with advanced disease showed improved survival in patients whose disease had become resistant to TAM, compared with patients treated with megesterol acetate (91-93). This led to further studies to compar the efficacy of AIs to that of TAM for the treatment of post-menopausal women with advanced BC. One study in over 900 patients demonstrated a significantly longer time to progression of 9.4 months in patients treated with the AI letrozole compared with 6 months for TAM-treated patients ($p < 0.0001$). The response rate using letrozole (32%) also compared favorably to that seen in patients treated with TAM (21%) (93; 94). Similarly, anastrozole and exemestane have proven superior to TAM in the metastatic setting (95-99).

Several large randomized trials in early BC with 5 years of TAM as the control arm which have examined the effectiveness of anastrozole, letrozole, and exemestane have generally found reduced recurrence rates compared with 5 years of TAM, none of the trials clearly demonstrated reduced BC mortality (100). This led to a collaboration through the Oxford Early Breast Cancer Trialists' Collaborative Group (EBCTCG) to pool data from all currently available trials of AIs versus TAM, using the methods of previous EBCTCG meta-analyses. The trials were grouped into two cohorts, which were analyzed separately. Cohort 1 consisted of trials comparing 5 years of an AI with 5 years of TAM, both starting soon after surgery (101; 102), while cohort 2 consisted of trials in which 2 to 3 years of TAM therapy was switched to 2 to 3 years of an AI compared with 2 to 3 more years of TAM (a total of 5 years of hormonal therapy in both groups) (103-105). Cohort 1 comprised 9,856 patients with a mean follow-up of 5.8 years. At 5 years, AI therapy was associated with a significant decrease in recurrence (9.6% for AI *vs.* 12.6% for TAM; $p<0.00001$) and a nonsignificant decrease in BC mortality (4.8% for AI *vs.* 5.9% for TAM; $p = 0.1$). Cohort 2 comprised 9,015 patients with a mean follow-up of 3.9 years. At 3 years from treatment divergence (i.e., approximately 5 years after starting hormone treatment), AI therapy was associated with a significant decrease in recurrence (5.0% for AI *v* 8.1% for TAM since divergence; $p<0.00001$) and a significant decrease in BC mortality (1.7% for AI *v* 2.4% for TAM since divergence; $p = 0.02$) (106).

Although AIs have been shown to be effective in the clinic, resistance to these therapies still occurs. Typical AI response rates vary from 20 to 50%. The exact reason for the lack of response or *de novo* resistance to AIs in some ER+ patients is not known. Although it is possible that acquired resistance results from aromatase or ER mutation developed during endocrine treatment, such resistance most likely results from cross-talk between ER and growth factor pathways or other currently unidentified pathways. Based on data on acquired resistance, almost all of which are currently derived from laboratory studies, it has been hypothesized that the adaptation to estrogen withdrawal is involved in resistance to AIs. Due to the

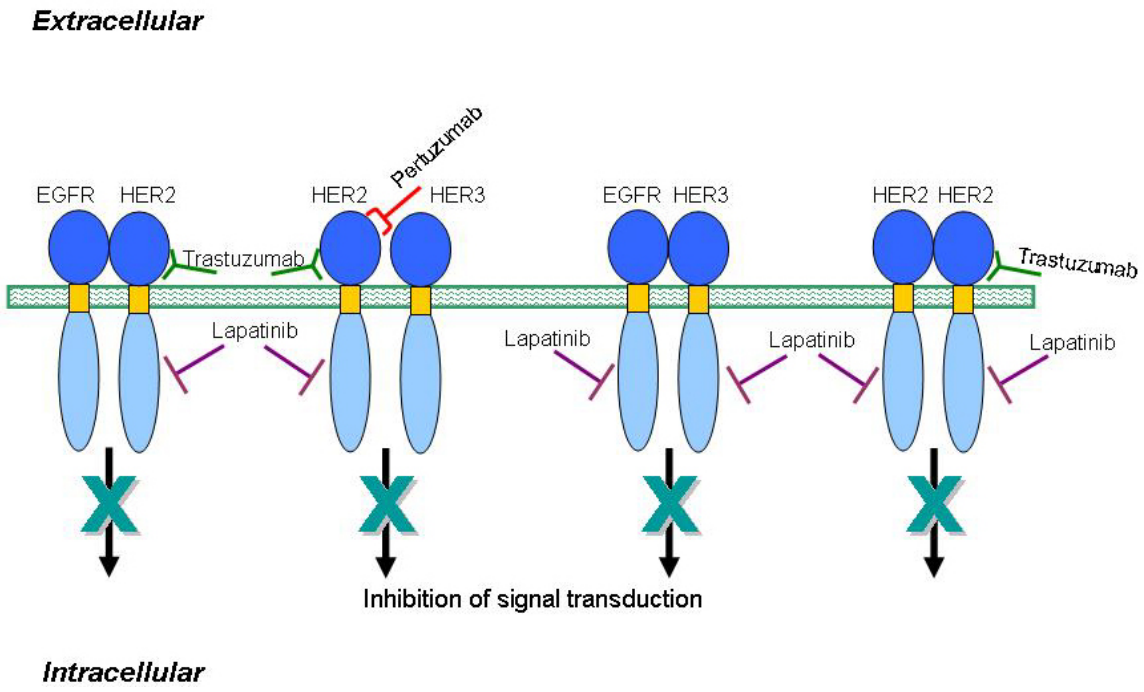

Figure 2. Anti-HER2 biodrugs. Binding sites of anti-HER2-directed current therapies.

adaptive ability of BC cells, this endocrine therapy may induce novel signaling mechanisms that circumvent the effects of an AI. Possibilities now under investigation using long-term estrogen-deprived cell lines (60; 107-109) are that resistance results from estrogen hypersensitivity or estrogen-independent activation of the ER. Findings in those studies have shown that growth factor pathways, i.e., HER2 and IGF1R, are activated in these estrogen-deprived cells, and that these receptors cross-talk with the ER, resulting in increased ER expression and phosphorylation, further activation of ER in a ligand-independent manner, and BC proliferation. Note that letrozole resistance has been shown to involve HER2 cross-talk with ER, leading to MAPK activation, ER phosphorylation, and ultimately BC cell proliferation (110; 111).

Because it remains possible that other currently unidentified pathways further augment AI resistance, methods such as cDNA microarray analysis have been applied to identify additional novel genes or pathways that might play a role in this resistance. Recent analysis of the transcriptome of 17 biopsies before treatment and 13 matched surgical samples of ER-positive breast tumors from patients after 3 months' treatment with the AI anastrozole indicated a decreased expression of cell proliferation-associated genes and an increased expression of inflammation-associated genes in the anastrozole-treated samples. Non-responders showed enrichment for genes associated with induction of T-cell anergy, positive regulation of androgen signaling, synaptic transmission and vesicle trafficking, while responders showed enrichment for cell cycle inhibition and induction of immune response markers. Furthermore, an expression signature of 54 genes predicted response in 100% of cases, and 5 of these genes accurately predicted response ($p = 0.0056$) in an independent dataset of 52 ER-positive BC treated with letrozole (112).

4. HER2-POSITIVE SUBTYPE

One of the most notable discoveries in BC translational research has been the identification of the HER2-positive BC subtype and the subsequent successful development of HER2-targeted therapies. Amplification of the HER2 oncogene is observed in approximately 25% of human BC and has been historically associated with poor prognosis. The HER2 receptor and other members of the EGFR family, i.e., HER1, HER3 and HER4 transmembrane kinases, are key regulators of signaling pathways that control numerous cellular functions, including proliferation, migration, survival, DNA repair and angiogenesis (113). Perturbation of HER2 signaling by genomic amplification causes the formation of activated homodimers or of heterodimers with other members of the family, mediating a downstream cascade of altered cellular signals through complex, interconnected, and still incompletely understood signal transduction pathways (113). The negative prognostic impact of HER2 gene amplification and/or oncoprotein overexpression has been greatly ameliorated by the development of HER2-targeted therapies. Two different types of HER2 inhibitors have been developed for clinical use: the humanized monoclonal antibody trastuzumab and the dual TKI lapatinib (Table 1).

4.1. Trastuzumab (T)

T is directed to an epitope in the cysteine-rich II domain of the extracellular domain of the HER2 receptor (114) (Figure 2) and was humanized at Genentech (South San Francisco, CA) by inserting its complementarity-

determining regions into the human immunoglobulin IgG1 framework (115). Preclinical studies to evaluate the therapeutic activity of this humanized antibody have demonstrated that T mediates at least antibody-dependent cell cytotoxicity (ADCC) and/or cytostatic activity by blocking HER2 proliferation pathways, and have indicated synergy or additivity between this antibody and several cytotoxic agents (116). In this context, a decisive trial designed to compare the effects of T plus anthracycline- or taxane-based chemotherapy with those of the same chemotherapy alone indicated a longer time to disease progression, a higher rate of objective response, and a reduced risk of death for patients with HER2-positive metastatic BC (MBC) when T was added to the chemotherapy protocol (117). Because of the survival benefit, T was approved in 1998 by the FDA for clinical treatment of women with HER2-positive MBC. The clinical benefits of T were found to be related to patients whose tumors show gene amplification, as detected by FISH and combined with intense immunohistochemical staining (score 3+) (118).

Because a survival benefit is rarely seen in randomized clinical trials for MBC, clinicians realized the great potential of T in improving cure rates of women with HER2-positive BC if used sooner in primary disease. In four multi-center trials, i.e., Herceptin Adjuvant (HERA), Breast Cancer International Research Group 006 (BCIRG 006), National Surgical Adjuvant Breast and Bowel Project (NSABP B-31) and North Central Cancer Treatment Group 9831 [NCCTG N9831) and two smaller clinical trials [Finnish Herceptin (FINHER) and Protocole Adjuvant dans le cancer du sein 04 (PACS 04)] of T in the adjuvant setting (119-125), all except for PACS 04 found that T reduced the risk of recurrence by 50% and the risk of death by about 33%, with consequent FDA/European Medicines Agency (EMEA) approval for T in early BC.

High pathologic complete response rates were also achieved in patients with HER2-positive BC treated with neoadjuvant T in combination with anthracyclines and taxanes. One-third of patients with significant residual disease lose HER2 amplification, a change associated with poor recurrence-free survival (126). Results from the GeparQuattro study (127) indicate that combining T with anthracycline- and taxane-based neoadjuavant chemotherapy leads to a high pathologic complete response without clinically relevant early toxicity. Further, the addition of neoadjuvant and adjuvant T to neoadjuvant chemotherapy has recently been suggested for women with HER2-positive locally advanced or inflammatory BC to improve event-free survival, overall survival, and clinico-pathological tumor responses (128).

While results thus far demonstrate the clinical benefit of T, the fact remains that this antibody administered according to current FDA/EMEA-approved protocols cures only about 50% of patients with HER2-positive early breast carcinoma and cannot cure those with HER2-positive MBC. Numerous *in vitro* studies have identified alterations downstream of the HER2 signaling pathway, such as PI3K mutation, pTEN knock-down and p27 decreased levels, overproduction of growth factors such as TGF-alpha and HB-EGF, and truncated p95 HER2 receptor as markers of T resistance (129-137), but none to date has proven sufficiently reliable *in vivo* to identify patients likely to be T-resistant. An explanation might reside in the apparently different operativity of the three major antitumor mechanisms of T, i.e., inhibition of proliferation, ADCC and, as recently proposed, inhibition of DNA repair due to its nuclear localization, in different therapeutic settings. Thus, definition of its mechanisms of clinical efficacy will be mandatory to improve the management of HER2-overexpressing BC. The studies on HER2-overexpressing metastatic disease indicate that T must be used as soon as possible and even continued at progression. Indeed, many clinical trials in patients with metastatic disease indicate that an HER2 blockade at any stage of disease progression is beneficial (138-140). These findings, indicating that the tumor maintains some sensitivity to anti-HER2 therapy even during relapse under T treatment, suggest that inhibition of tumor proliferation represents the mechanism by which the antibody restrains disease progression, although ADCC and inhibition of DNA repair might also play some role depending on the drugs combined with the antibody and on the patients' immune status.

Even in a small pilot study of T administered preoperatively as monotherapy, we found that patients who achieved complete or partial remission presented tumors with a higher *in situ* infiltration of leukocytes and with a higher ability to mediate *in vitro* ADCC (141; 142). ADCC is probably poorly active in patients due to chemotherapy-induced impairment of the immune system, but specific stimulation of ADCC effector cells by cytokines or by CpG-ODN should help in improving this effect.

In adjuvant trials, T has been limited to women whose tumors display HER2 amplification in light of results obtained in metastatic BC studies; however, women with BC that did not meet established criteria for HER2-positive carcinoma (i.e., HER2 score 2+/ FISH-negative) on central review were recently found to exhibit the same disease-free survival advantages as patients with HER2 score 3+ or FISH-positive tumors (143). Although these discrepancies might reflect errors in HER2 expression evaluation during the accrual of cases, consideration of the role of HER2 in regulating BC stem cells (144; 145) may provide an alternative biological explanation for a T-induced clinical benefit even in the absence of HER2 gene amplification. Our recent study (144) provided evidence of Notch-1-controlled enhanced expression of HER2 in BC stem cells, independent of HER2 gene copy number, as compared with levels in the cancer cell counterparts not expressing stemness markers. Thus, definition of the role of HER2 signaling in cancer stem cells of solid tumors can be useful in identifying molecular pathways that control cancer stem cells, leading to the development of efficient molecular-targeted strategies. Furthermore, the presence of HER2 in BC stem cells might also explain the T benefit on disease-free and overall survival even in the absence of tumor shrinkage.

T is generally well-tolerated, with mild-to-moderate side effects and a low incidence of chemotherapy-associated adverse events (117; 146-148). Infusion-related events such as fever and chills have been reported, but these are usually mild, restricted to the initial infusion and responsive to standard treatment. Cardiac effects represent the most relevant adverse events during T treatment, especially in women previously or concurrently exposed to anthracyclines (149). A recent meta-analysis to assess cardiac events associated with adjuvant T treatment for women with HER2-positive early BC found a favorable benefit:risk ratio for T (150); however, only longer follow-up of T-based trials will determine whether cardiac dysfunction has any long-term impact on patient outcome.

4.2. Lapatinib (L)

The small-molecule TKI L competes with ATP in the kinase domain of HER2 to impair the transmission of the proliferation signal (Figure 2). L, a dual TKI of both HER1 and HER2 (151; 152), blocks tumor cell proliferation by inhibiting phosphorylation of the receptors and preventing downstream signal transduction of PI3K and Ras. L has also been reported to have activity against T-resistant cells and to synergize with T in enhancing apoptosis in HER2-overexpressing cell lines (153; 154). Evidence for the efficacy of L in metastatic BC derives from trials of monotherapy involving patients with metastatic BC refractory to T or to anthracyclines, taxanes, or capecitabine plus T (155-158). The promising report by Gomez *et al* (158) of a 24% response rate to first-line L monotherapy in patients with HER2-positive BC or MBC led to evaluation of this drug in combination with capecitabine in HER2-positive metastatic BC cases refractory to regimens including an anthracycline, a taxane and T; the advantage of L plus capecitabine over capecitabine monotherapy was demonstrated, and fewer patients in the L arm developed central nervous system metastases, although the difference was not statistically significant (159). Based on those data, L was approved by the FDA and EMEA in 2007 for use in combination with capecitabine to treat patients with advanced or metastatic HER2-positive BC whose tumors progressed after anthracyclines, taxanes and T. Currently, L is being tested in the neoadjuvant and adjuvant settings. Based on results of a recent randomized trial (160) indicating that T plus L in HER2-positive metastatic BC patients progressing on T-based therapy was superior to the use of L alone, the Adjuvant Lapatinib and/or Trastuzumab Optimisation (ALTTO) trial has been initiated to compare effects of L alone, T alone, sequential use of both, and their combined use in HER2-positive early BC patients.

4.3. New therapeutic strategies

The complexity and interactions between HER2 and other signaling pathways provide clear rationale for targeting multiple pathways simultaneously. Targeting HER2 with multiple agents has been assessed. The monoclonal antibody pertuzumab, for example, binds to a distinct site from that of T binding on the HER2 extracellular domain and inhibits HER2-HER3 dimerization, thereby blocking multiple HER-mediated downstream pathways (161) (Figure 2). A phase II study of pertuzumab combined with T demonstrated an overall response rate of 24% in patients with T-resistant disease (162). A randomized phase III trial is underway to evaluate the combination of T, docetaxel and pertuzumab versus T and docetaxel alone as front-line therapy for metastatic BC (NCT00567190). Targeting multiple pathways simultaneously is also under investigation with a combination including T plus bevacizumab, an anti-VEGF monoclonal antibody that inhibits the angiogenesis necessary for tumor survival. A phase II study in patients with HER2-positive disease to assess T plus bevacizumab as first-line therapy for MBC (153) has provided promising interim efficacy data, with an objective response rate of 54.1%. The planned adjuvant BETH study (CIRG 011) will assess docetaxel, carboplatin, and T with or without bevacizumab in patients with HER2-positive early BC.

An alternative approach now being tested involves the fusion of a targeted agent and a cytotoxic agent in an effort to optimize delivery of chemotherapy to the tumor. T fused to the microtubule poison maytansine (DM1) is the first HER2 antibody-drug conjugate to be investigated for use in HER2-positive BC; in two phase I trials of patients with HER2-positive MBC who had progressed on prior T-based therapy, response rates of 44% and 53%, respectively, were observed, with no apparent cardiac-specific toxicity (163; 164) . The variability of clinical outcome and response of HER2-positive BC to anti-HER2 therapies points to the heterogeneous nature of this BC subset, consistent with a very recent unsupervised gene expression analysis of 58 HER2-amplified tumors that identified three separate subtypes with different clinical behavior (165). Further analysis of these gene expression data identified a 158-gene prognostic predictor (HDPP gene signature) that significantly improved stratification of patients with poor and good prognosis. The prognostic value of the HDPP signature was also shown for BLBCs but not for the luminal subtypes (165).

5. BASAL-LIKE SUBTYPE (BLBC)

It is estimated that 3 to 15% of all BC originate from basal-like epithelium and express basal-specific cytokeratins (CKs), such as CKs 5/6, 14, and 17. Most BLBCs are immunohistochemically negative for ER, PgR and HER2 and thus designated as "triple-negative" BCs (TNBCs). However, there is moderate discordance between TNBCs and BLBCs (166). This heterogeneous group of BC also overexpresses HER1 and c-kit in 45 to 75% of cases, and is associated with a higher rate of BRCA1 and p53 mutations than the other BC subtypes (167). Histologically, the majority of TNBCs are grade III invasive ductal carcinomas of no special type, although most medullary, metaplastic and adenoid cystic carcinomas also display a TN phenotype (168). TNBCs are more prevalent in young women (<50 years) of African and Hispanic descent. Multiple datasets have consistently identified a poorer clinical outcome for women with BLBCs and many investigations have collectively raised the possibility that this subtype comprises separate diseases rather than a single heterogeneous entity (169). In the absence of an established therapeutic biotarget, conventional cytotoxic

therapies are the current mainstay of treatment for these patients (169). A retrospective evaluation of the CALGB 9344 trial revealed that patients with TNBCs derived the greatest benefit from the addition of paclitaxel to doxorubicin and cyclophosphamide (170). However, it should be noted that while women with primary BLBCs appear to be more likely to respond to chemotherapy, their prognosis is worse if the tumor is not chemosensitive, given the reliance on chemotherapy alone in the absence of known targetable molecules. Interestingly, recent clinical trial data indicate that adjuvant tandem high-dose chemotherapy may be more effective than standard-dose therapy in improving 5-year event-free and overall survival in patients with the TNBC phenotype (171). TNBCs are known to have reduced levels of the DNA repair protein BRCA1, increasing their sensitivity to the DNA-damaging effects of platinum compounds (172). Further improvements in this type of therapy are expected from an ongoing phase III trial to compare the efficacy of carboplatin versus docetaxel in patients with metastatic TNBC.

The promise of a targeted approach in this subtype of BC is real, particularly with anti-angiogenic agents, such as bevacizumab, which impairs VEGF binding to VEGFR-2 and blocks downstream signals to disrupt angiogenesis. Originally FDA-approved for first- or second-line treatment of metastatic colorectal and non-squamous non-small cell lung cancers when given in combination with chemotherapy, bevacizumab has been recently approved by the FDA for use in MBC in association with paclitaxel for treatment of patients with no prior chemotherapy for metastatic HER2-negative BC (173). Interim findings from an ongoing phase III trial to study the efficacy of bevacizumab combined with the widely used taxane docetaxel (174) show that addition of bevacizumab to taxanes, and also to doxorubicin and capecitabine, improves progression-free survival in the first-line metastatic setting. Nevertheless, no study thus far has demonstrated an overall survival benefit from this approach, suggesting that bevacizumab slows tumor growth but does not extend survival, further highlighting the importance of continued research into inhibition of angiogenesis (173). Bevacizumab is well tolerated and when combined with taxanes, does not impact greatly on the known safety profile of these agents; adverse effects are generally manageable and are not more common in the bevacizumab arms than in the placebo arm (174).

Other compounds are being tested for treatment of TNBCs in the effort to pair the molecular biology of TNBCs with drug mechanisms. A recent study of the multi-kinase VEGFR inhibitor sunitinib indicated a response rate of about 15% in the pretreated TN subset of patients (175). In light of EGFR expression in this subset, several groups have also examined EGFR-targeted reagents in TNBCs. For example, the TBCRC 001 randomized phase II study of eligible patients who received the anti-EGFR monoclonal antibody cetuximab alone or in combination with carboplatin found a low response rate with cetuximab alone but a 17% response rate to the combination of cetuximab plus carboplatin, with clinical benefit in 29% of a pretreated population (176). Other novel agents of interest include the multi-targeted Src-Abl inhibitor dasatinib as well as inhibitors of polyADP-ribose polymerase (PARP), a nuclear enzyme involved in nonhomologous DNA repair and activated by the damage caused by chemotherapy and/or radiotherapy. Targeting PARP may prevent tumor cells from repairing DNA, especially in tumors with reduced BRCA1 levels, and thus may prevent drug resistance and even enhance tumor cell sensitivity to cancer therapies (177; 178). Several PARP inhibitors are currently being investigated in phase II trials, including the current multicenter randomized phase II trial of the PARP inhibitor BSI-201 alone or in combination with gemcitabine plus carboplatin in 120 patients with metastatic TNBC for which final analysis is expected by the end of 2010. Very recently, Turner *et al.* (179) used integrative molecular profiling of TNBCs to identify amplicon drivers and potential therapeutic targets and, in turn, to discover a peculiar subset (4%) of TNBCs consistently overexpressing fibroblast growth factor receptor 2 and thus providing a potential biotarget. The urgent need to define specific markers for TNBCs has led to the initiation of several trials targeting the TNBC subset and aimed at identifying which subgroups will benefit most from a specific drug in the next few years.

6. PERSPECTIVE

The advent of therapies based on mechanisms that target critical pathways of breast carcinomas has delivered promising clinical results, notably in patients with ER- or HER2-expressing BC in metastatic or adjuvant setting. An emerging role for therapies targeting angiogenic pathways is also now becoming recognized. Nevertheless, the development of targeted therapies remains challenging since breast tumors can present many potential targets but no obvious critical molecular drivers. Moreover, growing clinical and biological evidence indicates that tumor cells can develop unexpected mechanisms of resistance to these targeted therapies. Understanding such underlying mechanisms remains a goal and may lead to an exciting array of therapeutic strategies for women with refractory diseases. The growing challenge for clinical researchers is the appropriate identification of patients with molecular alterations likely to benefit from novel molecular approaches. It is noteworthy that despite many years of intense investigations, currently only ER and HER2 are universally accepted by the scientific community as predictive factors for hormone therapy and HER2-targeted therapies. Unfortunately, these two distinct biomarkers alone do not provide an accurate picture of BC aggressiveness and clinical outcome. Only with the advances achieved in molecular medicine and molecular oncology it has been and will continue to be possible to optimize prognosis, prediction and therapy of BC and to evaluate gene expression in tumors on a genome-wide basis rather than the single-gene level. To fulfill the promise of tailored BC care, an improved understanding of the biological and functional behavior of individual tumors is prerequisite for "improved stratification" of patients who require additional treatment. Future studies are also necessary to determine the optimal combinations, doses,

and schedules of therapies required to maximize clinical activity while minimizing toxicity.

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