Target-based therapies in breast cancer: current status and future perspectives

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Abstract

Identification of molecular alterations in key proteins involved in breast cancer cell proliferation and survival resulted in the development of a new treatment strategy with target-based agents. The anti-ErbB-2 monoclonal antibody (mAb) trastuzumab and the dual epidermal growth factor receptor/ErbB-2 tyrosine kinase inhibitor lapatinib are effective in patients with breast cancer that overexpresses ErbB-2. The anti-vascular endothelial growth factor-A mAb bevacizumab is approved in combination with taxanes for treatment of unselected patients with metastatic breast cancer. In addition, preclinical data suggest that signaling inhibitors can prevent or overcome resistance to endocrine therapy in estrogen receptor positive (ER+) breast cancer. However, the majority of signaling inhibitors explored in breast cancer patients has shown little activity, at least when used as monotherapy; and the results of clinical trials in ER+ breast cancer of combinations of signaling inhibitors and endocrine therapies are rather disappointing. Negative findings are likely due to mechanisms of intrinsic or acquired resistance to target-based agents. Breast carcinoma is a complex and heterogeneous disease and several different molecular alterations are involved in its pathogenesis and progression. The redundancy of oncogenic pathways activated in cancer cells, the heterogeneity of the mechanisms of resistance, and the plasticity of tumor cells that are capable to adapt to different growth conditions, significantly hamper the efficacy of each signaling inhibitor in breast cancer. Therefore, a comprehensive approach that takes into account the complexity of the disease is definitely required to improve the efficacy of target-based therapy in breast cancer.

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Introduction

The identification of mechanisms that regulate proliferation and survival of tumor cells is leading to the development of novel therapeutic approaches. To date, several agents specific for molecular targets amplified or overexpressed in cancer cells have been generated. Owing to their high selectivity, these drugs generally have fewer side effects as compared with most conventional chemotherapeutic agents, and can be combined with conventional therapies to improve the response to treatment without a major increase in side effects.

Breast cancer is a complex and heterogeneous disease. The development of high throughput technologies has recently underscored such complexity by revealing the existence of different subtypes of breast carcinoma that are characterized by specific gene expression profiles (Sorlie et al. 2001). As a matter of fact, endocrine treatment of estrogen receptor positive (ER+) breast cancer with tamoxifen, and later on with aromatase inhibitors and fulvestrant, has been the first target-based therapeutic strategy in oncology (Normanno et al. 2005c,d). The anti-ErbB-2 monoclonal antibody (mAb) trastuzumab and, more recently, the dual EGFR/HER2 tyrosine kinase inhibitor (TKI) lapatinib have shown significant clinical activity in patients with breast cancer that overexpresses the ErbB-2 receptor (Nanda 2007). However, some other
innovative approaches, including drugs directed against angiogenesis, have been developed in unselected patients because of the lack of information on the role of the specific signal transduction pathways in the pathogenesis and progression of the different subtypes of breast cancer.

This review aims to provide an overview on current status and future perspectives of target-based therapies in breast cancer. Before discussing the preclinical and clinical findings obtained with these agents, we will briefly describe the main signaling pathways against which target-based agents are being developed in breast cancer.

**Molecular targets in breast cancer**

The growth and survival of breast cancer cells is sustained by different growth factor receptor-driven signaling pathways (Fig. 1). Among these, the role of the epidermal growth factor receptor (EGFR) family of tyrosine kinase receptors in the pathogenesis of breast cancer has long been established. The EGFR family includes four different receptor tyrosine kinases: EGFR (ErbB-1), ErbB-2 (HER2), ErbB-3, and ErbB-4 (Normanno et al. 2005a). Each of these proteins possesses an extracellular ligand-binding domain, a single hydrophobic transmembrane domain and a cytoplasmic tyrosine kinase-containing domain (Olayioye et al. 2000). The receptors of the ErbB family are activated following binding to peptide growth factors of the EGF-family that induce formation of either homo- or hetero-dimers. Dimer formation precedes the activation of the kinase that leads to an auto- and trans-phosphorylation in tyrosine residues (Olayioye et al. 2000).

Expression of the EGFR has been reported in 14–91% of breast carcinomas (Salomon et al. 1995, Normanno et al. 2003). Overexpression of the EGFR has been linked to a more aggressive breast tumor phenotype and to poorer patient prognosis, although the results are discordant (Salomon et al. 1995, Normanno et al. 2003, 2005a,c). More recently, it has been shown that the ‘triple negative’ breast cancer subtype expresses the EGFR at higher frequency as compared with other subtypes (Reis-Filho & Tutt 2008). Expression of ErbB-2 is more restricted and occurs in ~20–30% of human primary breast carcinomas. High levels of expression of this receptor generally correlate with poor prognosis, although mixed results have also been reported (Salomon et al. 1995, Normanno et al. 2003, 2005a). ErbB-3 and ErbB-4 expression has been demonstrated to occur at high frequency in breast cancer patients (Salomon et al. 1995, Normanno et al. 2003, 2005a).

**Figure 1** Growth factor receptor-driven signaling pathways and target-based agents in clinical development in breast cancer. Binding of specific ligands that are produced by either tumor cells or by surrounding stromal cells activate growth factor receptors expressed by tumor cells, including ErbB receptors. The activated tyrosine kinase receptors are able to interact with signaling molecules that regulate different mechanisms involved in tumor pathogenesis and progression, such as proliferation, survival, invasion, and angiogenesis. VEGF receptors mediate the downstream effects of VEGF, which leads to the activation of intracellular signaling transduction pathways that are involved in endothelial cell proliferation, migration, and survival.
Co-expression of two or more ErbB receptors has been frequently found in breast carcinoma (Normanno et al. 2003, 2005a,c). Expression of phosphorylated ErbB-2 or co-expression of ErbB-2 and EGFR was associated with shorter survival in breast cancer patients (DiGiovanna et al. 2005). Similarly, co-expression of EGFR, ErbB-2, and ErbB-3 had negative synergetic effect on patient outcome, independent of tumor size or lymph node status (Wiseman et al. 2005). The redundancy of expression is not limited to the ErbB receptors but it also occurs for EGF-like peptides, such as transforming growth factor-α (TGF-α), amphiregulin and/or neuregulin(s) that are expressed at high frequency in primary breast tumors (Normanno et al. 2001). Finally, ErbB receptors and EGF-like peptides are generally expressed at higher levels in ER− breast carcinomas as compared with ER+ tumors (Normanno et al. 2001). However, a progressive increase in the levels of expression and activation of EGFR and ErbB-2 has been described in ER− breast cancer cells that develop resistance to anti-estrogen therapy (Nicholson et al. 2004, Normanno et al. 2005c,d).

Following ligand-induced activation, the tyrosine-phosphorylated receptors become able to interact with adaptor proteins that couple the receptors to intracellular signaling pathways (Olayioye et al. 2000). The ErbB receptors can activate different intracellular signaling cascades, including the phosphatidylinositol 3-kinase (PI3K)/v-akt murine thymoma viral oncogene homolog 1 (AKT) and the Ras/Raf/mitogen-activated protein kinase kinase (MEK)/mitogen-activated protein kinase (MAPK) pathways. However, these pathways might also be activated in an ErbB-independent manner, by molecular alterations of signaling proteins or by tyrosine kinase receptors other than the ErbB receptors.

The Ras/Raf/MEK/MAPK pathway is activated by tyrosine kinase receptors through either Grb2 and Sos or Shc adaptor proteins (Downward 2003). In turn, Ras activates Raf that, through intermediate steps, leads to phosphorylation of p42/44 MAPK (Downward 2003). Several studies have demonstrated that MAPK signaling promotes proliferation and survival of breast cancer cells (Dunn et al. 2005). Furthermore, activation of MAPK signaling has been associated with resistance to both EGFR targeting agents and endocrine therapy in breast carcinoma (Normanno et al. 2005c,d, 2006). Mutations of Ras and B-Raf genes that lead to abnormal activation of this pathway have been rarely identified in human primary breast cancer (Bos 1989). Surprisingly, Ras mutations have been recently described in 18% of human breast cancer cell lines (Hollestelle et al. 2007). Similarly, B-Raf mutations have been identified in 10% of breast cancer cell lines (Hollestelle et al. 2007). These findings suggest that Ras or Raf mutations might occur in a late stage of breast tumor progression, and that they might provide a growth advantage to clones of cells that can generate continuous cell lines.

The protein kinase C (PKC) family consists of at least 12 serine/threonine kinases that mediate intracellular signaling (reviewed in Mackay & Twelves 2007). Phosphorylation of tyrosine kinase receptors, such as the EGFR and the vascular endothelial growth factor receptor (VEGFR), induces activation of PKC through phospholipase Cγ. The downstream events following PKC activation are little understood, although both the MEK/MAPK and the PI3K/AKT pathways are thought to have an important role. Activated PKC phosphorylates and activates a range of kinases. Among these, the serine/threonine kinase glycogen synthase kinase 3β (GSK3β) is involved in metabolism, development, and apoptosis and is also one of the main targets of the PI3K/AKT pathway. Furthermore, PKCs α, β, and η directly phosphorylate AKT. It has been suggested that PKC plays a role in the pathogenesis of breast cancer. The total levels of PKC enzymatic activity are elevated in malignant breast tumors when compared with normal breast tissue (O’Brian et al. 1989, Gordge et al. 1996). The PKCβ isoform is known to be an important mediator of angiogenesis and represents an emerging target in breast cancer (Sledge & Gokmen-Polar 2006).

The PI3K/AKT pathway regulates different functions that play an important role in tumor progression, such as cell growth, survival, invasion, and migration (Liu et al. 2007). A mechanism for abnormal PI3K activation in cancer is through somatic mutations in the genes that encode positive and negative effectors of this pathway (Crowder & Ellis 2005). Loss of expression or functional loss of PTEN, a powerful negative regulator of PI3K signaling, occurs in different cancer types and results in constitutive AKT activation (Ali et al. 1999, Vivanco & Sawyers 2002). The frequency of PTEN mutations in human primary breast carcinoma is ~6% (Forbes et al. 2006). More recently, activating mutations of the PIK3CA gene, which encodes for the PI3K p110 catalytic subunit, were found in ~25% of primary breast tumors (Karakas et al. 2006).

One of the main targets of the PI3K/AKT cascade is the serine/threonine kinase mammalian target of rapamycin (mTOR), which belongs to the phosphoinositide kinase-related kinase family (Liu et al. 2007). Activation of mTOR, in turn, regulates translation initiation through activation of ribosomal p70S6 kinase (S6K1) and inactivation of the 4E-BP1.
suppressor protein (Liu et al. 2007). The RPS6KB1 gene, which encodes S6K1, is amplified in ~10% of breast cancer (Sinclair et al. 2003). RPS6KB1 gene amplification correlates with ErbB-2 overexpression in breast tumors, possibly due to coamplification of RPS6KB1 with ErbB-2 (Sinclair et al. 2003).

Increasing evidence suggests a role of Src in breast cancer progression. Src is the prototype of a large family of nonreceptor protein tyrosine kinases, known as the Src family kinases (Yeatman 2004). Src can be activated by cytoplasmic proteins, such as focal adhesion kinase (FAK) or its molecular partner Crk-associated substrate, which is involved in integrin signaling, and by ligand-activated tyrosine kinases of cell surface receptors, including EGFR and ErbB-2 (Yeatman 2004). Src is able to activate several different intracellular signaling pathways, including the PI3K/AKT and the Ras/Raf/MAPK pathways. In human mammary carcinomas a Src kinase activity 4- to 20-fold higher than normal tissues has been found (Irby & Yeatman 2000). A cooperation between Src and EGFR in breast cancer tumorigenesis has also been hypothesized (Maa et al. 1995, Dimri et al. 2007). Finally, Src plays an important role in epithelial to mesenchymal transition (EMT) that enhances the metastatic potential of tumor cells (Larue & Bellacosa 2005).

Angiogenesis, the formation of new blood vessels from the existing vasculature, is essential for the growth of the primary tumor and for the formation of metastasis. One of the key molecules responsible for the regulation of tumor-associated neoangiogenesis is VEGF-A (from now referred as VEGF), although additional growth factors, such as interleukin-8, basic fibroblast growth factor and the EGFR ligands EGF and TGF-α, might play a role in this phenomenon (Ferrara & Kerbel 2005, Kowanetz & Ferrara 2006). VEGF binds two related receptor tyrosine kinases: VEGFR-1 (Flt-1) and VEGFR-2 (Flk/KDR; Ferrara & Kerbel 2005). VEGFR-1 is a potent, positive regulator of physiologic and developmental angiogenesis and is involved in endothelial cell migration and differentiation. VEGFR-2 mediates the majority of the downstream effects of VEGF, including vascular permeability, endothelial cell proliferation, invasion, migration, and survival. A third member of the VEGFR family, VEGFR-3, is involved in lymphangiogenesis. Increased VEGF expression has been observed in breast cancer patients (Schneider & Miller 2005). Several studies have suggested that a correlation might exist between high VEGF expression and poor clinical outcome and lack of response to tamoxifen and chemotherapy in patients with advanced breast cancer (Schneider & Miller 2005).

Target-based agents in breast cancer

The target-based agents that have been approved for therapy of breast cancer patients or that entered clinical development can be divided in three large groups:

1) agents directed against specific subtypes of breast cancer, such as anti-estrogen (that will not be discussed in this paper) and anti-ErbB-2 compounds;
2) drugs targeting the tumor microenvironment such as anti-angiogenic agents that are potentially active in all the different subtypes of breast carcinoma;
3) inhibitors of specific signaling pathways, for the majority of which a role in the treatment of specific subtypes of breast carcinoma has not been demonstrated yet.

Anti-ErbB-2 drugs

In the past two decades several agents directed against the ErbB receptors have been developed. They include mAbs that bind the extracellular domain of the target receptor, and small molecule TKIs, which directly inhibit tyrosine kinase phosphorylation by physical interaction with either the ATP and/or the enzyme substrate binding site (Table 1; Normanno et al. 2003).

Trastuzumab

Trastuzumab is a humanized mAb with high specificity for ErbB-2 that showed moderate clinical activity in first or second-line treatment of ErbB-2 positive metastatic breast cancer as single agent (Baselga et al. 1999, Cobleigh et al. 1999, Vogel et al. 2002) or in combination with chemotherapy (reviewed in Demonty et al. (2007)). The addition of trastuzumab to first-line chemotherapy (either paclitaxel or anthracycline based) significantly improved response rate, time to progression (TTP), and overall survival in a pivotal randomized phase III trial (Slamon et al. 2001; Table 2). The benefit of combination treatment was also confirmed by a quality of life analysis (Osoba et al. 2002). The high rate of cardiotoxicity in the subgroup of patients treated concurrently with anthracyclines limited the use of this latter combination in clinical practice.

Two randomized phase II studies confirmed the efficacy and the safety of the combination of trastuzumab with weekly paclitaxel or 3-weekly docetaxel (Marty et al. 2005, Gasparini et al. 2007). However, due to an increasing use of taxanes in the
adjuvant setting, alternative trastuzumab-based combinations were developed, such as combinations with vinorelbine (Burstein et al. 2001, 2003, Papaldo et al. 2006, De Maio et al. 2007) and capecitabine (Bartsch et al. 2007, Schaller et al. 2007). Combinations of trastuzumab with polychemotherapy have been also studied. Regimens including a taxane and a platinum salt (Burris et al. 2004, Pegram et al. 2004, Robert et al. 2006) showed high response rates, but induced severe nonhematologic toxicities, including fatigue, nausea, vomiting, and neurotoxicity, which limit their use in clinical practice of metastatic breast cancer where palliation is the goal of treatment. An active triplet for patients previously treated with taxanes is the combination of trastuzumab with gemcitabine and vinorelbine that produced a response rate of 50% as second line therapy (Morabito et al. 2006b). However, there is yet no prospective demonstration of convenience of three-drug versus two-drug trastuzumab-based combinations in metastatic breast cancer.

An open question is the opportunity of continuing trastuzumab in combination with a non cross-resistant chemotherapeutic regimen in patients who progress on trastuzumab. Preclinical observations support the use of trastuzumab treatment beyond progression (Fujimoto-Ouchi et al. 2005). Until recently, clinical evidences in contrast or in favor of this hypothesis came only from retrospective (Fountzilas et al. 2003, Gelmon et al. 2004, Tripathy et al. 2004, Bartsch et al. 2006, Montemurro et al. 2006) or phase II studies (Bartsch et al. 2007, 2008). At ASCO 2008, two phase III trials have been reported. In the one not yet published in extenso (O’Shaughnessy et al. 2008), trastuzumab was added to lapatinib and compared with lapatinib alone, as salvage treatment in 296 patients; a very small advantage was shown in TTP (median 2.8 vs 1.9 months), that was statistically significant \((P<0.029)\) but does not represent a clinically significant progress, with no advantage in response rate and survival. The other phase III study (von Minckwitz et al. 2009) assessed the efficacy of continuing trastuzumab combined with capecitabine compared with capecitabine alone in patients progressing <6 weeks since the end of the last trastuzumab cycle. The study was planned to detect a prolongation of 1 month in TTP, and required 482 patients, with the first interim analysis planned after 150 events. However, it was performed after 82 events and, although the Independant Data Monitoring Committee suggested to continue the study, it was stopped with 156 randomized patients because of slowing accrual. TTP was slightly prolonged in the combination arm (median 8.2 vs 5.6 months, \(P=0.034)\) and response rate was increased (48 vs 27%, \(P=0.0068)\) but no significant difference was found in survival. Overall, the level of evidence actually available in favor of continuing trastuzumab beyond

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CSF-1R, colony-stimulating factor 1 receptor; FLT3, FMS-like tyrosine kinase 3; PDGFR, platelet-derived growth factor receptor; PxF, human peroxisomal farnesylated protein.

Table 1 Target-based agents in clinical development in breast cancer

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<table>
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<tr>
<th>Agent</th>
<th>Author (year)</th>
<th>Study phase</th>
<th>Line</th>
<th>No. of patients</th>
<th>Arms</th>
<th>RR (%)</th>
<th>TTP (months)</th>
<th>P</th>
<th>OS (months)</th>
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<td>1-2-3</td>
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<td>versus Cetuximab + Carboplatin</td>
<td>17</td>
<td>2</td>
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<td>versus Paclitaxel + Carboplatin</td>
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<td>3-4</td>
<td>296</td>
<td>versus Capecitabine</td>
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<td>versus Lapatinib</td>
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<td>a</td>
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<td>versus Paclitaxel</td>
<td>21.2</td>
<td>5.9</td>
<td>a</td>
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<td>Capecitabine + Bevacizum versus Bevacizum</td>
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<td>0.857</td>
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<td>Miller et al.</td>
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<td>versus Capecitabine</td>
<td>9.1</td>
<td>4.17</td>
<td>a</td>
<td>14.5</td>
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<td>Miles et al.</td>
<td>(2008)</td>
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<td>1</td>
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<td>&lt;0.001</td>
<td>26.7</td>
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<td>5.9</td>
<td>a</td>
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<td>versus Paclitaxel + Bevacizum (7.5 mg/kg)</td>
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<td>versus Docetaxel + Placebo</td>
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<td>Phase II</td>
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<td>Docetaxel + Axitinib versus Docetaxel + Placebo</td>
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<td>versus Docetaxel + Placebo</td>
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<td>Phase II</td>
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<td>141</td>
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<td></td>
<td></td>
<td></td>
<td>versus Lapatinib</td>
<td>22.2</td>
<td>NR</td>
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RR, response rate; TTP, time to progression; OS, overall survival; NR, not reached.

aProgression-free survival.
bPretreated with trastuzumab.
progression remains weak, because of limitations of studies performed, and it suggests that the advantage deriving from continuing trastuzumab might be very small, if any.

The evidence of a molecular cross-talk between ER and the ErbB-2 pathway, and the observation that ErbB-2 overexpression is associated with preclinical and clinical resistance to hormonal therapy, suggested that combining treatments targeting both pathways may provide additional benefits for breast cancer patients (Nicholson et al. 2004, Normanno et al. 2005c,d). A phase III trial (TANDEM) investigated the efficacy of adding trastuzumab to anastrozole alone as first-line therapy in postmenopausal patients with ER and ErbB-2 positive metastatic breast cancer (Mackey et al. 2006). The results of this trial led to the extension of trastuzumab indication thanks to a significant improvement in progression free survival (4.8 vs 2.4 months) and response rate (20.3 vs 6.8%; Table 3). However, the very poor outcome even in the winner arm prevents to consider this combination as a reasonable standard of treatment. In addition, the hypothesis that endocrine treatment is not effective at all and that better trastuzumab-based combinations might be used in this subgroup of patients is not ruled out by the TANDEM study. Two phase III trials are evaluating the effect of adding trastuzumab to letrozole or tamoxifen in ER/ErbB-2 positive patients.

Some large international randomized phase III clinical studies demonstrated the efficacy of adjuvant trastuzumab in women with ErbB-2 positive early breast cancer (Table 4); unfortunately, some of them have not been published in extenso, although being presented in 2005 for the first time (N9831) or repeatedly in subsequent years (BCIRG 006; Slamon et al. 2006). Despite differences in the patient population and design, all these studies showed similar results, namely a significant improvement of disease-free survival, with a hazard ratio (HR) of 0.46–0.67 for women receiving trastuzumab, with the exception of PACS 04 trial, an underpowered study where a 0.86 HR was found (Spielmann et al. 2007). Some trials also demonstrated a prolongation of overall survival. The standard duration of adjuvant trastuzumab treatment is now 1 year, based on HERA and Joint Analysis (NSABP B-31 and NCCTG N9831) results (Piccart-Gebhart et al. 2005, Romond et al. 2005, Smith et al. 2007a). However, it might be not the optimal one. The HERA trial will compare 2 years with 1 year of trastuzumab. On the other hand, the FinHer trial showed that 9 weeks of trastuzumab given concurrently with three cycles of docetaxel or vinorelbine and followed by three cycles of fluorouracil, epirubicin and cyclophosphamide are enough to significantly improve disease-free survival (Joensuu et al. 2006). Two further randomized phase III trials are testing the efficacy of a shorter duration of adjuvant trastuzumab: the Short-HER Trial (NCT00629278), an Italian trial of two different adjuvant chemotherapy regimens plus 3 vs 12 months of trastuzumab, and the PHARE study (NCT00381901) that is currently comparing 6 with 12 months of adjuvant trastuzumab.

Another unanswered question is whether giving trastuzumab as sequential or partially combined with chemotherapy does produce different efficacy. The only direct comparison currently available, in the NCCTG N9831 trial (Perez 2005), suggests that giving trastuzumab concomitantly with paclitaxel for 3 months and then as single agent up to 1 year is more effective than giving it sequentially after chemotherapy. Finally, the unpublished BCIRG 006 suggests the possibility that a non-anthracycline containing regimen (docetaxel and carboplatin plus trastuzumab) might be effective approximately as a sequential scheme with AC followed by docetaxel plus trastuzumab. This might represent a useful therapeutic opportunity in patients not candidate to anthracyclines. Efficacy of adjuvant trastuzumab is still unknown among patients with very small tumors (<1 cm) and among those who do not receive adjuvant chemotherapy.

**Lapatinib**

Lapatinib is an oral, dual TKI of both EGFR and ErbB-2. In preclinical studies, lapatinib produced growth inhibition in a variety of human tumor cell lines overexpressing either EGFR or ErbB-2, including breast cancer (Rusnak et al. 2001). Thanks to its ability of binding also with the truncated form of ErbB-2 (p95 ErbB-2) that lacks the extracellular domain, lapatinib might be effective in ErbB-2 positive tumors that are resistant to trastuzumab (Scaltriti et al. 2007). Following early clinical trials (Blackwell et al. 2005, Burris et al. 2005, Spector et al. 2005, Burstein et al. 2008b), a phase III registrative trial, in patients with ErbB-2 positive metastatic breast cancer who had received multiple previous treatments, demonstrated that the addition of lapatinib to capecitabine significantly prolonged TTP (Geyer et al. 2006; Table 2), with no difference in overall survival. The latter result might be jeopardized by crossover that was allowed after the anticipated study closure due to the positive results at an interim analysis of TTP. Treatment was well tolerated and the rate of adverse events was similar in the two arms, the main difference being an
<table>
<thead>
<tr>
<th>Agent</th>
<th>Author</th>
<th>Study phase</th>
<th>Line</th>
<th>No. of patients</th>
<th>Arms</th>
<th>RR (%)</th>
<th>PFS (months)</th>
<th>OS (months)</th>
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<td>Trastuzumab</td>
<td>Mackey et al. (2006)</td>
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<td>1</td>
<td>207</td>
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<td>56</td>
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<td>54</td>
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<td>Smith et al. (2007a,b)</td>
<td>Phase II</td>
<td>Preoperative</td>
<td>206</td>
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<td>48</td>
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<td>61</td>
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<td>Cristofanilli et al. (2008)</td>
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<td>versus anastrozole + placebo</td>
<td>34</td>
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<td>Osborne et al. (2007)</td>
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<td>versus tamoxifen + placebo</td>
<td>14.9</td>
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<td>Phase II</td>
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<td>Johnston et al. (2008a,b)</td>
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<td>Letrozole + tipifarnib</td>
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<td>versus letrozole + placebo</td>
<td>38</td>
<td>10.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NR</td>
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RR, response rate; PFS, progression-free survival; OS, overall survival; NR, not reached.

<sup>a</sup>Not pretreated with aromatase inhibitors.
<sup>b</sup>Pretreated with aromatase inhibitors.
<sup>c</sup>Time to progression.
increase of grade 1 and 2 diarrhea in the combination arm (45 vs 28%). Another phase III trial assessed the worth of adding lapatinib to chemotherapy, namely paclitaxel, in a population of 580 patients with ErbB-2 negative/untested advanced breast cancer (Di Leo et al. 2007). The result was negative, with no difference in TTP, the primary endpoint, except for a small subgroup of 86 patients whose tumor resulted to be ErbB-2 positive at pathology revision, where the addition of lapatinib prolonged TTP. Similar results have been recently reported in the phase III EGF30008 study testing the efficacy of lapatinib added to endocrine treatment with letrozole (Johnston et al. 2008a). The rationale of this study was based on preclinical studies showing that the combination of lapatinib with anti-estrogens might delay or prevent the development of resistance to lapatinib in ErbB-2-overexpressing/ER-positive breast cancer cells and might overcome endocrine resistance (Chu et al. 2005, Xia et al. 2006). Lapatinib plus letrozole significantly prolonged PFS as compared with letrozole alone among postmenopausal patients with ER-positive tumors. The benefit, however, seems to be limited to patients with ErbB-2 positive metastatic breast cancer, and is not evident among those with ErbB-2 negative tumors. All the above evidences, considered together, strongly support the hypothesis that lapatinib therapeutic effect is prevalently limited to patients with ErbB-2 positive breast cancer.

Promising preliminary results have been reported with lapatinib as single agent. A phase II study of lapatinib monotherapy in relapsed or refractory inflammatory breast cancer showed, in ErbB-2 positive patients, a response rate of 62%, with additional 21% of the patients experiencing stabilization of disease. In contrast, only 8.3% of EGFR positive/ErbB-2 negative patients achieved a partial response (Spector et al. 2006). Biomarker analyses of tumor biopsies showed that co-expression of phospho-ErbB-2 and phospho-ErbB-3 was predictive of lapatinib response. Usefulness of lapatinib in an earlier phase of breast cancer is not definite, yet. Two randomized phase III studies, the ALTTO and NeoALTTO trials, are evaluating its efficacy as single agent or in combination with trastuzumab as compared with trastuzumab as single agent in the adjuvant and neoadjuvant treatment of ErbB-2 positive breast cancer patients.

Because of the unexpected cardiac toxicity evidenced in trastuzumab trials, great attention has been given to cardiac safety of lapatinib. A review of cardiotoxicity data of nearly 3000 patients enrolled in 18 phase I–III clinical trials, including 1674 breast cancer patients.
cancer patients, treated with lapatinib alone or in combination with other agents, reported an incidence of symptomatic or asymptomatic decline in left ventricular ejection fraction of 1.3% (Perez et al. 2006). Lapatinib does not appear to increase the risk of cardiomyopathy, even when combined with trastuzumab (Storniolo et al. 2007).

**Pertuzumab**

Pertuzumab is a humanized recombinant mAb directed against the extracellular dimerization domain of the ErbB-2 receptor. This antibody directly inhibits the dimerization of the ErbB-2 protein with other ErbB family receptors, preventing the activation of downstream signaling pathways. The different and potentially complementary mechanism of action is the rationale for associating pertuzumab with trastuzumab (Nahta et al. 2004). These two mAbs have been combined in a phase II study, in 66 heavily pretreated patients affected by locally advanced or metastatic ErbB-2 positive breast cancer, whose disease had progressed during trastuzumab therapy (Gelmon et al. 2008). Frequent toxicities included diarrhea (63%), pain (35%), nausea/vomiting (30%), mucositis (32%), and skin rash (26%). A response rate of 18.2% and a long lasting stabilization of disease in 21.2% of patients were observed. Randomized phase II and phase III trials are evaluating the effectiveness of pertuzumab in combination with trastuzumab and chemotherapy as first-line therapy in metastatic disease.

**Anti-angiogenic agents in breast cancer**

Anti-angiogenic agents in advanced phase of clinical development in breast cancer include neutralizing antibodies against VEGF (bevacizumab) and TKIs of VEGFRs (sorafenib, sunitinib, vandetanib, axitinib, pazopanib; Table 1).

**Bevacizumab**

Bevacizumab is a humanized mAb that binds VEGF and prevents its interaction with VEGF receptors, thus leading to inhibition of VEGF-induced angiogenesis. Bevacizumab can be administered safely, without dose-limiting toxicities, up to the dose of 10 mg/kg every 2 weeks, and can be combined with chemotherapy without apparent synergistic toxicity (Gordon et al. 2001, Margolin et al. 2001). In pretreated metastatic breast cancer bevacizumab has very limited activity as single agent (Cobleigh et al. 2003) and, when added to capecitabine, produced an increase in response rates that did not translate into improved PFS or overall survival in a randomized phase III trial (Miller et al. 2005a).

On the contrary, the addition of bevacizumab to first-line chemotherapy has been found effective in two phase III trials (Table 2). In the E2100 trial, bevacizumab plus paclitaxel significantly prolonged progression-free survival (median 11.8 vs 5.9 months; HR of progression 0.60, P < 0.001), and increased the objective response rate (36.9 vs 21.2%) of 722 patients with metastatic breast cancer as compared with paclitaxel alone, although there was no advantage in survival (Miller et al. 2007). In the AVADO trial, 736 patients with ErbB-2 negative metastatic breast cancer were randomized to receive docetaxel plus bevacizumab (either the dose of 7.5 or 15 mg/kg) or docetaxel alone (Miles et al. 2008). At both doses, bevacizumab in combination with docetaxel significantly improved progression-free survival (HR 0.69 and 0.61 respectively) and response rate (55.2% and 63.1 vs 44.4% respectively), as compared with docetaxel alone. Combination treatment was well tolerated at both doses of bevacizumab.

Preclinical studies have shown that initial events in the development of metastasis are VEGF-dependent, suggesting that angiogenesis inhibitors might be more effective in the adjuvant setting. The ECOG E5103 trial will evaluate the efficacy of the addition of bevacizumab to four cycles of AC followed by 12 courses of weekly paclitaxel and the role of bevacizumab as maintenance therapy. In the BEATRICE trial, standard adjuvant chemotherapy will be compared with chemotherapy plus bevacizumab for 1 year in early triple negative breast cancer. Finally, the BETH study will evaluate the efficacy of the combination of adjuvant bevacizumab, trastuzumab and chemotherapy versus chemotherapy plus trastuzumab in ErbB-2 positive breast cancer patients.

In the neoadjuvant setting, preliminary safety results are available for the combination of bevacizumab and docetaxel, showing a good tolerability of such treatment (Lyons et al. 2006). Bevacizumab was also tested in combination with doxorubicin and docetaxel as neoadjuvant treatment of women with inflammatory breast cancer (Wedam et al. 2006). Fourteen of 21 enrolled patients experienced a clinical partial response and showed a decrease in vascular permeability on dynamic contrast-enhanced magnetic resonance imaging that was consistent with reduced angiogenesis. Moreover, this study indicated a potential direct anti-tumor effect of bevacizumab, as
suggested by increased apoptosis and decreased phosphorylated VEGFR2 in tumor cells. However, the role of VEGFR2 in the survival of breast cancer cells has not been formally proven yet. A pilot trial of neoadjuvant letrozole in combination with bevacizumab in postmenopausal women with newly diagnosed operable breast cancer showed an overall response rate of 74% (Forero-Torres et al. 2008).

Sorafenib

Sorafenib is an oral multikinase inhibitor with activity on cancer cell proliferation and angiogenesis (Wilhelm et al. 2004). Sorafenib inhibits Raf kinase isoforms (Raf-1, wild-type B-Raf, and mutant B-Raf) and Raf-dependent activation of MAPK pathway in breast cancer cells that carry mutations of both K-Ras and B-Raf (Wilhelm et al. 2004). Sorafenib also inhibits several receptor tyrosine kinases involved in angiogenesis such as VEGFR-2, VEGFR-3, platelet-derived growth factor receptor (PDGFR)-β, and stem-cell factor receptor (c-KIT). The inhibition of receptor tyrosine kinases autophosphorylation occurs at significantly lower drug concentration as compared with the blockade of the Raf/MEK/MAPK pathway, which might require inhibition of multiple Raf isoforms (Wilhelm et al. 2004). Once-daily oral dosing of sorafenib demonstrated broad-spectrum anti-tumor activity in colon, breast, and non-small-cell lung cancer xenografts (Wilhelm et al. 2004). Inhibition of the Raf/MEK/MAPK pathway was demonstrated in some but not all models, whereas a significant reduction of neovascularization was found in all the xenograft models, suggesting that both mechanisms contribute to the growth inhibitory activity of sorafenib (Wilhelm et al. 2004).

Negative results were reported with sorafenib as single agent in patients with metastatic breast cancer (Moreno-Aspitia et al. 2006). In this study, higher circulating ErbB-2 levels and/or higher baseline serum VEGF levels were associated with shorter TTP. Studies of sorafenib in combination with chemotherapeutic agents in metastatic breast cancer patients are ongoing.

Sunitinib

Sunitinib is an oral multitarget receptor TKI of VEGFR-1, VEGFR-2 and VEGFR-3, PDGFRs-α and -β, c-KIT, glial cell line-derived neurotrophic factor receptor (rearranged during transfection; RET), FMS-like tyrosine kinase 3, and colony-stimulating factor 1 receptor (Chow & Eckhardt 2007).

In breast cancer tumor xenograft models, sunitinib demonstrated a significant anti-tumor activity (Abrams et al. 2003). Moreover, activity of sunitinib has been demonstrated in an in vivo model of breast cancer bone metastases and tumor-associated osteolysis (Murray et al. 2003). Interestingly, a recent report showed that short-term treatment with sunitinib or with other VEGFR-TKIs (sorafenib and SU10944) produced an increase in tumor growth and metastatization in different orthotopic mice models including breast cancer, suggesting that the schedule and the duration of treatment might significantly affect the efficacy of these drugs (Ebos et al. 2009).

A phase II study of sunitinib (50 mg/day, 4 weeks on/2 weeks off) in patients with anthracycline and taxane-resistant metastatic breast cancer showed a response rate of 11% (Burstein et al. 2008a). Interestingly, among the ‘triple negative’ patients, the response rate was 15%, while in the ErbB-2 positive, trastuzumab pretreated patients, the response rate was 25%. The most frequently reported adverse effect was fatigue, followed by nausea, diarrhea, mucosal inflammation, and anorexia. However, a randomized phase III trial evaluating single-agent sunitinib versus single-agent capecitabine for the treatment of patients with advanced breast cancer after failure of standard treatment was recently discontinued for futility. Phase II and phase III clinical trials of sunitinib in combination with chemotherapeutic agents (capecitabine, docetaxel, paclitaxel) or target-based agents (bevacizumab or trastuzumab) are ongoing.

Vandetanib

Vandetanib is a small molecule receptor TKI that inhibits VEGFR-2, EGFR, and RET and that has shown anti-tumor activity in a broad range of preclinical models (Wedge et al. 2002, Ciardiello et al. 2003, 2004). Phase I studies of vandetanib in patients with advanced solid tumors have demonstrated that the once-daily oral administration at 100–300 mg/day is well tolerated (Tamura et al. 2006). However, the drug showed limited activity in patients with metastatic breast cancer, who had received prior treatment with an anthracycline and taxanes, as no objective response was observed and only one patient experienced stable disease ≥24 weeks (Miller et al. 2005b). A randomized phase II study of the combination of vandetanib (100 mg daily) plus docetaxel (100 mg/m² 3 weeks) versus docetaxel plus placebo as second-line treatment for advanced breast cancer failed to demonstrate any advantage for the combination (Table 2; Boer et al. 2007). An Italian randomized
phase II trial (the ZACFAST study) will compare two doses of vandetanib (100 and 300 mg) versus placebo in combination with fulvestrant, an ER antagonist with no estrogen agonist effects, in postmenopausal patients with endocrine responsive metastatic breast cancer (Morabito et al. 2009).

Axitinib

Axitinib is an oral potent inhibitor of VEGFRs 1, 2, and 3 (reviewed in Morabito et al. 2006a). Preclinical studies demonstrated that axitinib blocks angiogenesis and tumor blood flow in tumor models (Inai et al. 2004). A phase I trial identified axitinib 5 mg twice daily as the recommended clinical dose; hypertension, hemoptysis, and stomatitis were dose-limiting toxicities (Rugo et al. 2005). A randomized, double-blind phase II study evaluated the activity of the combination of axitinib (5 mg twice daily) and docetaxel (80 mg/m²) compared with docetaxel plus placebo as first-line treatment of 168 patients with metastatic breast cancer (Table 2; Rugo et al. 2007). The combination had an acceptable safety profile, but showed only a modest improvement in TTP (8.2 vs 7.0 months, P=0.052). In a predefined subgroup analysis, TTP was increased by the addition of axitinib to docetaxel only in patients that were previously treated with adjuvant chemotherapy (9.0 vs 6.3 months, P=0.012).

Pazopanib

Pazopanib is an oral small molecule multitargeted receptor TKI of VEGFR-1, -2, -3, PDGFR-α, PDGFR-β, and c-kit tyrosine kinases (Kumar et al. 2007). Maximum tolerated dose (MTD) was not achieved in a phase I study, but the recommended phase II dose was defined at 800 mg/day (Hurwitz et al. 2005). The most common adverse events were nausea, hypertension, diarrhea, fatigue, anorexia, vomiting, and hair depigmentation. A randomized phase II study evaluated the combination of pazopanib and lapatinib versus lapatinib alone as first-line therapy of 141 patients with ErbB-2 positive metastatic breast cancer (Table 2; Slamon et al. 2008). The combination of pazopanib and lapatinib showed an increased response rate when compared with lapatinib monotherapy (36.2 vs 22.2%), but no difference in progression rate at week 12, the primary endpoint of the study. Ongoing studies are evaluating the activity of pazopanib in combination with lapatinib in inflammatory breast cancer, with exemestane in postmenopausal ER+ breast cancer or as single agent in patients with metastatic breast cancer pretreated with chemotherapy.

Inhibitors of signaling pathways

A number of agents directed against different signaling pathways have been developed. As we will discuss, the majority of these agents have been explored in unselected breast cancer patients and have shown little or no activity. However, preclinical and clinical findings suggest a potential role of some of these agents in specific settings.

Anti-EGFR agents

Gefitinib

Gefitinib is an oral EGFR TKI. In preclinical studies, this drug inhibited the growth of a wide range of EGFR-expressing human cancer cell lines, including breast cancer cells (Moasser et al. 2001, Moulder et al. 2002, Campiglio et al. 2004).

Several phase II clinical trials with gefitinib as single agent in breast cancer patients have been reported (Normanno et al. 2005c,d). These studies showed no significant clinical activity in pretreated advanced breast cancer patients (Albain et al. 2002, Robertson et al. 2003, Baselga et al. 2005, von Minckwitz et al. 2005). Similarly, addition of gefitinib to chemotherapy did not result in an improvement of anti-tumor activity (Fountzilas et al. 2005, Ciardiello et al. 2006).

Pharmacodynamic studies in breast cancer patients treated with gefitinib showed that EGFR phosphorylation was successfully inhibited in tumor biopsies in all patients, suggesting that resistance to gefitinib is due to lack of tumor dependence upon EGFR (Baselga et al. 2005). In agreement with this hypothesis, preclinical studies showed that EGFR-independent activation of either MAPK or AKT signaling might mediate resistance to gefitinib (Moasser et al. 2001, Moulder et al. 2001, Normanno et al. 2006). Activation of the insulin-like growth factor type 1 receptor (IGF-1R), a tyrosine kinase receptor that is a powerful activator of PI3K/AKT signaling, has also been associated with both de novo and acquired resistance to gefitinib (Jones et al. 2004, Camirand et al. 2005). More recently, it has been shown that breast cancer cells that co-express EGFR, ErbB-2, and ErbB-3, such as SK-Br-3 cells, escape the activity of EGFR-TKIs through ErbB-2-dependent activation of the ErbB-3/PI3K/AKT pathway (Sergina et al. 2007). However, this mechanism of resistance developed following short treatment with EGFR-TKIs (up to 96 h). When SK-Br-3 breast cancer cells were exposed for a long term (5–8 months) to the drug, a persistent EGFR-independent activation of MAPK and increased sensitivity to MEK inhibitors were observed (Normanno et al. 2008).
Preclinical studies also support the use of EGFR-TKIs in combination with anti-hormonal agents. Increased levels of expression of EGFR and/or ErbB-2 and increased sensitivity to anti-EGFR agents have been found in breast cancer cells with acquired resistance to tamoxifen, letrozole, or fulvestrant, as well as in estrogen-dependent cells cultured for long term in absence of estrogen, an in vitro condition that resembles anti-estrogen therapy in vivo (Knowlden et al. 2003, Martin et al. 2003). In addition, it has been shown that treatment of breast cancer cells with combinations of anti-EGFR and/or anti-ErbB-2 drugs and endocrine agents prevents the occurrence of resistant clones (Gee et al. 2003, Xia et al. 2006). However, mixed results have been obtained in clinical trials.

Two studies of preoperative gefitinib in combination with anastrozole showed contrasting results (Table 3). In a randomized phase II clinical trial, 56 patients with ER+/EGFR+ primary untreated breast cancer were treated with gefitinib and anastrozole or gefitinib plus placebo (Polychronis et al. 2005). Ultrasonography revealed a significant decrease of tumor mass in 50% of patients treated with gefitinib and anastrozole, and in 54% of patients that received gefitinib as single agent. Negative results were found in a neoadjuvant randomized phase II clinical trial (Smith et al. 2007b), in which 206 patients were randomized to receive anastrozole plus gefitinib or anastrozole plus placebo for 2 weeks followed by gefitinib for 14 weeks or anastrozole plus placebo for 16 weeks. Neither biological nor clinical activity of anastrozole was enhanced by the addition of gefitinib. However, it must be emphasized that this trial did not require EGFR positivity as inclusion criteria.

No clinical benefit was found with gefitinib plus anastrozole in patients with ER+ advanced breast cancer, who had previously failed hormonal therapy (Mita et al. 2005), while a significant increase in median progression free survival (14.5 vs 8.2 months) was seen with the same combination as first-line treatment of metastatic breast cancer (Cristofanilli et al. 2008). Finally, the results of a randomized phase II trial of gefitinib or placebo in combination with tamoxifen in patients with ER+/PR+ metastatic breast cancer showed a modest advantage in progression free survival with the combination in women with newly diagnosed cancer or who had completed adjuvant tamoxifen from at least 1 year, but not in patients previously treated with aromatase inhibitors (Table 3; Osborne et al. 2007).

Overall, these data suggest that gefitinib might enhance the activity of anti-estrogen therapies by preventing the resistance to endocrine therapy. However, this phenomenon is likely to occur in specific subsets of patients that develop an EGFR-dependent mechanism of resistance. Indeed, different molecular mechanisms seem to be involved in the resistance to endocrine therapy, as we will discuss in the next paragraphs.

Finally, gefitinib has been described to have activity on bone pain in selected breast cancer patients (Albain et al. 2002, von Minckwitz et al. 2005). The mechanism involved in this phenomenon has not been clarified yet. However, we have shown that gefitinib affects the ability of bone marrow stromal cells to secrete pro-osteoclastogenic factors and to induce osteoclast differentiation, thus suggesting a specific activity of this agent on the pathogenesis of bone metastases (Normanno et al. 2005b).

Erlotinib

Erlotinib is an oral reversible inhibitor of the EGFR tyrosine kinase, which has shown a potent anti-tumor activity in preclinical studies (Normanno et al. 2003). Erlotinib is also able to block the ErbB-2 kinase in intact cells through direct interaction with ErbB-2 (Schaefer et al. 2007). However, this drug is 12-fold less active against ErbB-2 kinase as compared with the EGFR kinase. A pharmacodynamic study showed that treatment of patients with operable breast cancer (stage I–IIIA) with erlotinib (150 mg/day) for 6–10 days before surgery produced a significant reduction of the activation of both EGFR and ErbB-2 (Guix et al. 2008). Erlotinib also induced a statistically significant reduction of AKT and MAPK phosphorylation and suppressed ER phosphorylation at ser118 in ER+ breast tumors. A complete cell cycle response, as defined by a <1% Ki67 index after neoadjuvant therapy, was observed in 17 patients, 16 of which were ER+. Little activity in metastatic breast cancer patients has been shown with erlotinib as single agent (Dickler et al. 2009) and in combination with letrozole (Mayer et al. 2006).

Cetuximab

Cetuximab is a chimeric human–mouse mAb that binds competitively to the extracellular domain of the EGFR, inhibiting its autophosphorylation and inducing its internalization and degradation. In preclinical studies, cetuximab showed anti-tumoral activity against a variety of human tumor xenografts and displayed synergistic effects when used with classical cytotoxic agents and radiation (Normanno et al. 2003, Ciardiello & Tortora 2008).
A dose-escalation phase I trial of cetuximab and paclitaxel, as first or second line of treatment, in patients with EGFR-positive advanced breast cancer evidenced a prohibitive dermatologic toxicity and low activity (Modi et al. 2006). A single arm trial evaluating cetuximab in combination with irinotecan in anthracyclines and taxane-pretreated patients with metastatic breast cancer was closed prematurely due to low activity, although a clinical benefit rate of 27% was reported in patients with triple negative tumors. (Hobday et al. 2008). In a randomized phase II trial in this latter subset, the combination of cetuximab and carboplatin resulted in a response rate of 18%, but most of the patients progressed rapidly (Carey et al. 2008; Table 2). Negative results were also reported with the combination of cetuximab with irinotecan and carboplatin as first- or second-line therapy of metastatic breast cancer patients, although it resulted in activity in the subgroup of the triple negative breast cancer patients (O’Shaughnessy et al. 2007).

**mTOR inhibitors**

The mTOR pathway plays a central role in the regulation of cell growth, proliferation, and survival. Although neither mTOR mutations nor its overexpression have been reported in human tumors, signaling pathways that modulate mTOR are frequently deregulated in human cancers, including breast cancer. Rapamycin, the prototype of mTOR inhibitors, inhibited tumor growth in a wide range of experimental malignancies (Bjornsti & Houghton 2004). Temsirolimus and everolimus are the rapamycin analogues currently in clinical development (Table 1).

**Temsirolimus**

Temsirolimus is a water-soluble ester of rapamycin, which demonstrated anti-tumor activity in a variety of cancer models (Yu et al. 2001). Breast cancer cell lines sensitive to temsirolimus were estrogen dependent, or lacked expression of PTEN and/or overexpressed ErbB-2, while resistant lines shared none of these properties (Yu et al. 2001). AKT resulted highly activated in sensitive but only minimally in resistant cell lines. A synergistic effect of the combination of temsirolimus with endocrine therapy has also been shown (deGraffenried et al. 2004). Finally, preclinical findings revealed that temsirolimus has an antiangiogenic activity by inhibiting VEGF production in tumor cells and by affecting endothelial cell proliferation and morphogenesis (Del Bufalo et al. 2006). A pharmacodynamic study showed that temsirolimus inhibited pS6K activity in peripheral blood mononuclear cells (PBMCs) and tumor tissue in vivo in a parallel fashion, indicating that the PBMCs could be an appropriate surrogate tissue (Peralba et al. 2003).

A randomized phase II study explored the anti-tumor activity of two doses of temsirolimus (75 and 250 mg/week i.v.), in 109 heavily pretreated patients with locally advanced/metastatic breast cancer (Chan et al. 2005). A response rate of 9% and a median TTP of 12 weeks were reported in the intent-to-treat population. Efficacy was similar for both dose levels but toxicity was more common with the higher dose. Preliminary data from a randomized phase II trial of letrozole with or without oral temsirolimus as first or second line of treatment of locally advanced or metastatic breast cancer showed that the combination treatment resulted well tolerated at the dose of temsirolimus of 10 mg daily or 30 mg for 5 days every 2 weeks (Carpenter et al. 2005; Table 3). However, a large randomized, placebo-controlled, double-blind phase III trial exploring the efficacy of the combination of letrozole plus temsirolimus as first-line hormonal therapy was closed on the basis of data from a planned interim analysis, showing that the trial was unlikely to achieve the targeted level of efficacy for the combination therapy compared with letrozole alone (Chow et al. 2006).

**Everolimus**

Everolimus is an oral mTOR inhibitor under clinical evaluation in different types of cancer. In preclinical models everolimus enhanced growth inhibition by trastuzumab and gefitinib in PTEN-deficient cells, overcoming resistance to these drugs (Lu et al. 2007, Bianco et al. 2008). Everolimus also inhibited estrogen-driven proliferation and increased the activity of letrozole in estrogen-dependent MCF-7 breast cancer cells (Boulay et al. 2005).

A pharmacodynamic phase I study showed that inhibition of mTOR signaling occurred at all dose levels and schedules in tumor and skin biopsies, being almost complete at 10 mg daily and 50 mg weekly (Taberner et al. 2008). The most frequent toxicities were skin rash, stomatitis, headache, and fatigue. In a phase II neoadjuvant trial, everolimus (5 mg daily for 14 days) produced a significant decrease of Ki67 labeling index and phospho-AKT (pAKT) levels in 30 patients with breast cancer (Macaskill et al. 2006). High pretreatment pAKT correlated significantly with greater reductions in proliferation.

A phase II double-blind, randomized, placebo-controlled trial, evaluated the value of adding everolimus to letrozole as preoperative therapy of primary
breast cancer in postmenopausal women (Baselga et al. 2009; Table 3). The study showed a higher response rate by palpation (primary endpoint) in the combination arm (68.1 vs 59.1%) that was also confirmed by ultrasound (58 vs 47%). A biomarker analysis indicated a significant down-regulation between baseline and day 15 tumor biopsies for pS6K in response to everolimus (Gardner et al. 2007). Cell cycle response, as defined by proportion of patients with Ki67 ≤2 at day 15, was also significantly higher in the combination arm (57 vs 30%, P<0.01). Most frequent severe toxicities related to everolimus were hyperglycemia, stomatitis, interstitial lung disease/pneumonitis, and infections.

Farnesyl transferase inhibitors
The most crucial modification involved in Ras activation is the covalent attachment of a farnesyl isoprenoid lipid to a cysteine residue in the COOH-terminal of Ras proteins that is catalyzed by the enzyme farnesyl transferase (Downward 2003). However, many potential substrates, independent of Ras, have been identified for farnesyl transferase inhibitors (FTIs), such as lamin A and human peroxisomal farnesylated protein, both of which have been used as surrogate markers of farnesylation, RhoB, cyclic guanosine monophosphate phosphodiesterase α, rhodopsin kinase and the γ subunit of the retinal protein, transducin (O’Regan & Khuri 2004). The oral FTIs currently in clinical development are tipifarnib, lonafarnib and AZD3409 (Table 1).

Tipifarnib
Preclinical studies have suggested a potential activity of tipifarnib in ER+ breast cancer cells (Kelland et al. 2001). In addition, a synergistic anti-tumor effect of combination of tipifarnib and 4-hydroxytamoxifen was observed in ER+ breast cancer cells (Martin et al. 2007).

Phase I studies of tipifarnib showed that continuous dosing was associated with severe toxicities, such as diarrhea, nausea, vomiting, renal dysfunction, and myelosuppression (O’Regan & Khuri 2004). A phase II study evaluated the activity of tipifarnib, as single agent, in endocrine- and/or chemotherapy-resistant patients with metastatic breast cancer (Johnston et al. 2003). A total of 76 patients were treated with tipifarnib, either as a continuous dose of 300 or 400 mg bis in die (BID; n=41) or an intermittent dose of 300 mg BID for 21 days followed by 7 days off therapy (n=35). In the continuous treatment arm, there were four partial responses (10%) and six patients with stable disease (15%) for at least 6 months. In the intermittent treatment arm, there were five partial responses (14%) and three patients with stable disease (9%). The incidence of hematological and neurological toxicities was significantly higher in the continuous treatment arm as compared with the intermittent arm. However, a randomized phase II trial of combination of tipifarnib and letrozole in postmenopausal women with ER+ advanced breast cancer showed negative results for the combination that did not improve patients outcome, but was more toxic in terms of asymptomatic grade 3/4 neutropenia (Johnston et al. 2008b; Table 3).

Lonafarnib
Lonafarnib has shown anti-tumor activity in different models of human xenografts (Liu et al. 1998). In phase I trials, the dose limiting toxicity (DLT) was reached at 300–400 mg BID, depending on the schedule (continuous vs intermittent) and it was usually gastrointestinal (O’Regan & Khuri 2004). Fatigue, which was severe at higher doses, and neutropenia were noted. Phase II studies of lonafarnib in combination with aromatase inhibitors (anastrozole) or trastuzumab plus chemotherapeutic agents (paclitaxel) are ongoing.

AZD3409
AZD3409 is a novel prenyl transferase inhibitor that was designed to mimic the C-terminal CAAX (CVIM: cysteine, valine, isoleucine, methionine) sequence of K-Ras 4B, the Ras isoform most commonly mutated in human cancers (Stephens et al. 2003, Wakeling 2005). This compound has shown activity against both farnesyl transferase and geranylgeranyl transferase I in isolated enzyme studies. AZD3409 was able to inhibit the growth of tumor cells carrying either a mutated or wild-type Ras gene (Stephens et al. 2003, Khafagy et al. 2004). Interestingly, in preclinical studies it has been demonstrated that AZD3409 significantly affected the growth of gefitinib-resistant breast cancer cells (Maiello et al. 2007). Results of a phase I trial of AZD3409 in patients with advanced solid malignancies defined the MTD at 750 mg BID in the fasted state (Appels et al. 2008). The dose-limiting toxicities were vomiting, diarrhea, and uncontrolled nausea. Pharmacodynamic studies also showed that farnesyl transferase was inhibited at all dose levels.

Src inhibitors
The role of Src in proliferation, invasion, angiogenesis, and metastasis supports the development of Src inhibitors.
inhibitors in breast cancer. In fact, blockade of Src activation may slow disease progression in the metastatic disease and prevent the formation of metastases in the adjuvant setting. In this regard, several inhibitors of Src are in clinical development in breast cancer (Table 1).

**Dasatinib**

Dasatinib is an oral small molecule kinase inhibitor of both Src and Abl proteins (Lombardo et al. 2004), actually registered in the treatment of leukemia. Dasatinib inhibited the growth of different breast cancer cell lines, showing higher activity in cell lines belonging to the basal-subtype or that have undergone EMT (Finn et al. 2007, Huang et al. 2007). A set of three genes, moesin, caveolin-1, and YAP-1, whose elevated expression is associated with response to dasatinib was identified (Finn et al. 2007). A six gene signature including caveolin 1 and 2, annexin A1, EPH receptor A2, polymerase I, and transcript release factor and IGF binding protein 2, was also found to predict sensitivity to dasatinib (Huang et al. 2007). Interestingly, this gene signature was observed in basal-like breast tumors, thus confirming the potential sensitivity of this subtype of breast carcinoma to dasatinib (Huang et al. 2007).

The results of a phase I clinical trial of dasatinib in solid tumors showed no dose-limiting toxicity with an oral dose of 35, 50, or 70 mg BID for 5 days followed by 2 days break, every week (Evans et al. 2005). A pharmacodynamic study showed that, on the BID regimen, Src phosphorylation was inhibited in a dose-dependent manner, by assessing Src activation in PBMCs (Luo et al. 2006).

**AZD0530**

AZD0530 is an oral anilinoquinazoline with a high affinity and specificity for the tyrosine kinase domain of Src and Abl, that are inhibited at low nanomolar concentrations of the drug (Hennequin et al. 2006). In preclinical studies, AZD0530 was able to inhibit the anchorage-independent growth of MCF-7 breast cancer cells wild-type or stably expressing a mutant ER with increased sensitivity to estrogen (Herynk et al. 2006). However, these effects were reversed by estrogen. A cooperative inhibitory effect was also observed when ER + breast cancer cells were treated with a combination of AZD0530 and tamoxifen, suggesting that therapeutic use of this drug in ER + breast cancer patients might require blockade of ER signaling (Herynk et al. 2006). AZD0530 also reduced motility and invasion of tamoxifen-resistant MCF-7 cells, and, in combination with tamoxifen, prevented the development of resistance to anti-estrogen therapy in MCF-7 cells (Hiscox et al. 2006). These findings provide a strong rationale for the development of AZD0530 in ER + breast cancer patients.

The results of a phase I and pharmacodynamic study of AZD0530 showed that the daily dose of 175 mg/day is the MTD (Tabernero et al. 2007). DLTs occurred in three patients at 250 mg (leukopenia, fatal septic shock with renal failure, asthenia) and in two patients at 200 mg (febrile neutropenia and dyspnea). A consistent modulation of phosphorylation and/or cellular localization of tumor paxillin and FAK, two targets of Src tyrosine kinase, were observed in patients treated with AZD0530. Finally, AZD0530 therapy produced a significant decrease in markers of osteoclast-mediated bone resorption with a dose–response trend.

**Bosutinib**

Bosutinib is an orally active inhibitor of Abl and Src kinases. Treatment of MDA-MB-231 breast cancer cells with bosutinib produced a marked inhibition of cell proliferation, invasion, and migration, as well as a significant inhibition of MAPK and AKT activation (Jallal et al. 2007). In a phase I study, the MTD was found at 500 mg/day once daily (Messersmith et al. 2007). However, the recommended dose for phase II studies was 400 mg, due to significant grade 2 gastrointestinal toxicity observed in patients treated with 500 mg. The most frequent adverse effects were nausea, diarrhea, anorexia, asthenia, and vomiting. Stabilization of the disease for more than 24 weeks was observed in 3/51 patients, including one breast cancer patient.

**Inhibitors of MEK signaling**

A number of MEK inhibitors are in preclinical and clinical development in different tumor types. Data in breast cancer are available only for AZD6244 (ARRY-142886), an highly specific MEK1/2 inhibitor that binds to the allosteric inhibitor-binding site and locks MEK1/2 in an inactive conformation (Table 1; Yeh et al. 2007). Preclinical studies have shown that tumor cells carrying activating Ras and/or B-Raf mutations are more sensitive to AZD6244 as compared with those possessing wild-type genes (Davies et al. 2007, Yeh et al. 2007). However, AZD6244 inhibited the in vivo growth of ZR-75-1 xenografts, which carry wild-type Ras and Raf genes, despite little effects on cell viability in vitro. The efficacy of AZD6244 in this tumor model might be due to inhibition of angiogenesis and/or
to increased dependency of tumor cells from MEK activation for their in vivo growth (Yeh et al. 2007).

A phase I clinical trial showed that AZD6244 orally was well tolerated up to 100 mg BID in patients with solid tumor, including breast cancer. The most frequent side effects were acneiform rash, diarrhea, nausea, and fatigue (Adjei et al. 2008). Complete inhibition of pMAPK phosphorylation in PBMCs was observed 1 h after drug administration and was maintained during therapy. Analyses of paired tumor samples collected before and after treatment showed that treatment with AZD6244 produced a significant inhibition of MAPK phosphorylation in tumor tissue. Ki-67 labeling index was also reduced in these samples but not as consistently as pMAPK. The best clinical response was stable disease, lasted for 5 or more months in 16% of patients. No correlation was found between activity of AZD6244 and mutational status of RAS or Raf.

Inhibitors of PKC

Enzastaurin is an orally available, potent inhibitor of PKCβ, which also inhibits other PKC isoenzymes, although at higher concentration as compared with PKCβ (Table 1; Graff et al. 2005). By blocking PKC activation, enzastaurin inhibits AKT and GSK3β activation, suppresses tumor cell proliferation, induces tumor cell death and inhibits VEGF-mediated angiogenesis. A phase I study showed that enzastaurin was well tolerated at doses up to 700 mg daily. No MTD was established and the recommended phase II dose was 525 mg/day, based on plasma levels inhibiting PKCβ (Carducci et al. 2006). Moreover, evidence of early activity in terms of stable disease was observed. However, no clinical activity was reported in a phase II study of enzastaurin as single agent in patients with metastatic breast cancer previously treated with anthracyclines and taxanes (Krop et al. 2008). Phase II studies are currently ongoing to evaluate the combination of enzastaurin with chemotherapy (capecitabine, paclitaxel), hormonal therapy (fulvestrant) or other targeted therapies (bevacizumab) in breast cancer.

Conclusions and future perspectives

The development of target-based agents in breast cancer has been characterized up to now by rather few successes and several failures. As matter of fact, the target-based agents that have shown significant clinical activity in breast cancer patients up to now are the anti-ErbB-2 drugs trastuzumab and lapatinib, and the anti-VEGF mAb bevacizumab. Interestingly, all these drugs have been approved for treatment of breast cancer patients in association with chemotherapy. Only trastuzumab and lapatinib have also shown activity as single agents.

It might be argued that target-based agents are active when their targets play a key role in tumor growth, such as ErbB-2 in breast tumors that overexpress this receptor. However, it must be emphasized that the majority of ErbB-2 positive patients have mechanisms of resistance to trastuzumab at diagnosis. In fact, only 30% of patients with advanced ErbB-2-positive breast cancer respond to trastuzumab as first-line therapy, and the response rate drops to 15% in pretreated patients. Furthermore, almost all patients will become resistant to the drug during the course of the treatment. Therefore, the main question that we need to address in order to improve this therapeutic approach is why tumors do not respond to target-based agents.

Cancer is the result of several, different genetic, and epigenetic alterations. Simultaneous activation of different oncogenic pathways has been shown in breast cancer cells, and these pathways are likely to cooperate in sustaining the proliferation and the survival of breast cancer cells. Resistance to treatment with target-based agents is, therefore, the consequence of the complex mechanisms that regulate the growth of breast tumors. It is possible that a minority of tumors depend on the activation of a single pathway (‘oncogenic addiction’), as shown for some ErbB-2 of ER positive tumors. However, evidence suggests that the majority of the tumors have a network of different aberrantly activated signaling pathways, and they unlikely will respond to the blockade of a single oncogene.

The activation of signaling pathways in breast cancer cells might be significantly altered by treatment with anti-cancer agents, including target-based agents and endocrine therapies. In fact, a number of studies have shown that breast cancer cells are able to escape the activity of target-based agents by developing adaptive mechanisms that lead to activation of alternative pathways involved in the proliferation and survival of tumor cells (Jones et al. 2004, Xia et al. 2006, Sergina et al. 2007, Normanno et al. 2008). The results of preclinical and clinical studies also suggest that heterogenous patterns of resistance might occur. For example, resistance to EGFR-TKIs in breast cancer has been linked to activation of ErbB-2/ErbB-3/AKT signaling, to persistent activation of MAPK or even to mutations of ErbB-2, depending on the background of the cell lines used, on the duration of exposure to the drugs and possibly other factors (Piechocki et al. 2007, Sergina et al. 2007, Normanno et al. 2008). The development of high throughput technologies is also
revealing that acquired resistance is frequently a complex phenomenon. Gene expression profiling of both fulvestrant- and tamoxifen-resistant breast cancer cells showed that resistance to endocrine therapy is characterized by significant up-regulation of multiple growth-stimulatory pathways (Fan et al. 2006). Finally, the pathways of resistance can change during the time. For example, tamoxifen-resistant breast cancer cells are extremely sensitive to gefitinib (Knowlden et al. 2003). However, prolonged exposure to gefitinib led to the development of resistance to this latter drug through the activation of an IGF-1R-dependent mechanism (Jones et al. 2004). Taken together, the above-mentioned findings suggest that the redundancy of oncogenic pathways activated in cancer cells, the heterogeneity of the mechanisms of resistance that have been found among primary tumors and cell lines, and the plasticity of tumor cells that are capable to adapt to different growth conditions, significantly hamper the efficacy of each signaling inhibitor in breast cancer (Fig. 2).

Increasing evidence suggests that cancer stem cells might be resistant to chemotherapy and therefore responsible for cancer relapse (Zhang & Rosen 2006). Recently, a neoadjuvant trial in breast cancer patients has shown that the percentage of putative cancer stem cells increased following treatment with chemotherapy, providing evidence supporting their intrinsic chemoresistance (Li et al. 2008). Interestingly, in the same trial it was found that lapatinib treatment of patients with ErbB-2 positive tumors did not lead to an increase in the percentage of cancer stem cells, suggesting that lapatinib treatment could eliminate ErbB-2 positive cancer stem cells. These preliminary observations support the hypothesis that addition of specific target-based agents to chemotherapy might lead to killing of cancer stem cells. In this regard, the molecular profiling of cancer stem cells will provide information that will be useful for the development of novel therapeutic strategies in which signaling pathways that are critical for their survival will be targeted.

The above-summarized information indicates that the molecular characterization of the tumors of each individual patient and the identification of biological markers that are associated with response or resistance to treatment are mandatory to improve the efficacy of target-based agents in breast cancer. An example comes, again, from lapatinib for which a six gene signature predictor of response to lapatinib has been identified (Gray et al. 2008). In addition, gene signatures that are correlated with the response/resistance to the src inhibitor dasatinib have been developed and might turn useful to select patients to be treated with this drug (Finn et al. 2007, Huang et al. 2007). Finally, gene signatures associated with the activation of specific signaling pathways are being developed, and they might allow in the future to identify the signaling pathways that are specifically activated in each individual tumor (Bild et al. 2006).

Although the majority of the target-based agents evaluated up to now failed to show significant clinical activity, it is likely that these agents might contribute to control breast cancer progression in the context of combinations of compounds targeting different signaling pathways that cooperate in sustaining the growth of breast tumor cells. Agents with a broader spectrum of action, such as histone deacetylase inhibitors and proteasome inhibitors that are in the initial phase of development in breast cancer, might also turn to be effective in this strategy. The high cost of target-based agents, the potential toxicity of their combinations and the complexity and relatively elevated costs of high-throughput technologies that are required to provide a molecular portrait of breast tumors are the limitations that scientists will have to face to develop this new therapeutic approach.

Declaration of interest

The authors declare no potential conflicts of interest.

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