Terms and Conditions for Use of PDF

The provision of PDFs for authors’ personal use is subject to the following Terms & Conditions:

The PDF provided is protected by copyright. All rights not specifically granted in these Terms & Conditions are expressly reserved. Printing and storage is for scholarly research and educational and personal use. Any copyright or other notices or disclaimers must not be removed, obscured or modified. The PDF may not be posted on an open-access website (including personal and university sites).

The PDF may be used as follows:
• to make copies of the article for your own personal use, including for your own classroom teaching use (this includes posting on a closed website for exclusive use by course students);
• to make copies and distribute copies (including through e-mail) of the article to research colleagues, for the personal use by such colleagues (but not commercially or systematically, e.g. via an e-mail list or list serve);
• to present the article at a meeting or conference and to distribute copies of such paper or article to the delegates attending the meeting;
• to include the article in full or in part in a thesis or dissertation (provided that this is not to be published commercially).
Overcoming Treatment Resistance in HER2-Positive Breast Cancer
Potential Strategies

Fabio Puglisi,1,2 Alessandro Marco Minisini,1 Carmine De Angelis3 and Grazia Arpino3
1 Department of Oncology, University Hospital of Udine, Udine, Italy
2 Department of Medical and Biological Sciences, University of Udine, Udine, Italy
3 Department of Clinical and Molecular Oncology and Endocrinology, University of Naples, Federico II, Naples, Italy

Abstract

Human epidermal growth factor receptor (HER)-2 overexpression or amplification occurs in about 20% of all breast cancers and results in a worse prognosis. Nevertheless, anti-HER2 treatments have recently been developed, resulting in dramatic improvements in the clinical outcome of patients with HER2-positive breast cancer. Trastuzumab has shown efficacy in early and advanced breast cancer treatment and lapatinib is currently approved for the treatment of advanced disease. Other anti-HER2 agents are being investigated. Mechanisms of resistance to trastuzumab treatment include crosstalk with heterologous receptors and amplification of HER2 signalling; amplification of the phosphoinositide 3-kinase (PI3K)/AKT pathway; alteration in binding of trastuzumab to HER2; and loss of HER2 expression. Proposed mechanisms of resistance to lapatinib involve derepression and/or activation of compensatory survival pathways through increased PI3K/AKT or estrogen receptor (ER) signalling. Several strategies to overcome resistance to anti-HER2 treatment are in different phases of development and include treatment with pertuzumab, T-DM1 and mammalian target of rapamycin (mTOR) inhibitors.

1. Introduction

In the past decades, insights into the genomic heterogeneity of breast cancer and the underlying biology have led to an individualized treatment approach due to the ability to identify in advance those patients who are most likely to respond to a particular therapy.1

Notably, in about 20% of all primary invasive breast cancers, amplification of the human epidermal growth factor receptor (HER)-2 proto-oncogene and/or overexpression of its protein is evident.2 These characteristics are associated with an increased propensity for metastases and clinically translate into reduced disease-free survival (DFS) and overall survival (OS).2-7 However, although HER2 positivity (i.e. gene amplification or protein overexpression) confers a poor prognosis to patients with such tumours, it predicts response to anti-HER2 targeted agents. In other words, HER2 positivity is an unfavourable prognostic factor and a favourable predictive factor at the same time.
Trastuzumab is a humanized IgG₁κ monoclonal antibody, specifically targeted against the extracellular domain (ECD) of HER2, where it binds with high affinity. In 1998, trastuzumab was approved in combination with chemotherapy for the treatment of HER2-overexpressing metastatic breast cancer (MBC), mainly based on the results of two randomized controlled trials (RCTs) showing a benefit in terms of OS with the addition of trastuzumab to chemotherapy. Unfortunately, although trastuzumab plus chemotherapy has improved the prognosis of patients with HER2-positive MBC, almost all patients eventually succumb to progressive disease, with a median OS of about 2–3 years in the majority of randomized trials.

In 2005, the efficacy of trastuzumab was also demonstrated in the adjuvant setting, where four randomized trials reported a significant reduction in the rates of recurrence and death in patients with HER2-positive early breast cancer (EBC) treated with trastuzumab and chemotherapy. Again, although it is anticipated that many patients treated with adjuvant trastuzumab will be cured of their disease, it is also expected that many will experience a recurrence.

Thus, a large proportion of patients with HER2-positive tumours either do not respond to trastuzumab or develop tolerance to the antibody, suggesting both de novo and acquired mechanisms of drug resistance.

Besides trastuzumab, other anti-HER2 agents (such as lapatinib) are currently available in the clinical setting, while others are still being developed in clinical trials.

This review focuses on potential mechanisms of resistance to anti-HER2 treatment, examining studies that have evaluated signalling from receptor tyrosine kinases (RTKs) outside of the HER family, increased phosphoinositide 3-kinase (PI3K) signalling, amplification of signalling by other receptors of the HER family and the presence of altered forms of HER2 that are not recognized or bound by trastuzumab. In addition, major results of clinical studies on novel anti-HER2 agents are also described in order to support the proof of concept that underlies the strategy to overcome resistance to trastuzumab.

2. State of the Art Treatment for HER2-Positive Breast Cancer

2.1 Early Disease

2.1.1 Adjuvant Treatment

In HER2-positive EBC, trastuzumab showed consistent results in four landmark phase III trials comparing trastuzumab-containing versus chemotherapy alone regimens. Both DFS and OS were significantly prolonged by 1 year of trastuzumab. At a median follow-up of 3.9 years, the joint analysis of the NCCTG N9831 (see table I for definitions of study names used in this article, where available) and NSABP B-31 trials showed a highly significant advantage in DFS (hazard ratio [HR] = 0.52, 95% CI 0.45, 0.6) and in OS (HR = 0.61, 95% CI 0.50, 0.75) for the trastuzumab-containing arms. Similarly, an updated analysis of the HERA trial at a median follow-up of 4 years confirmed the significant advantage in DFS for the trastuzumab-containing arm (HR = 0.76, 95% CI 0.66, 0.87) although the initial advantage in OS was not more statistically significant, mainly because of a high percentage of crossover.

In the BCIRG 006 trial, trastuzumab showed an improvement in DFS and OS even with a non-anthracycline-containing regimen (HR = 0.75, 95% CI 0.63, 0.90 and HR = 0.77, 95% CI 0.60, 0.99, respectively).

At present, the standard duration of adjuvant trastuzumab is 1 year, either in combination or after completing adjuvant chemotherapy.

The optimal duration of trastuzumab treatment is still under investigation. Results of the 2-year trastuzumab arm in the HERA trial are awaited.

Table 1. Trial acronyms and definitions

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALTTO</td>
<td>Adjuvant Lapatinib and/or Trastuzumab Treatment Optimisation</td>
</tr>
<tr>
<td>BCIRG</td>
<td>Breast Cancer International Research Group</td>
</tr>
<tr>
<td>CALGB</td>
<td>Cancer and Leukemia Group B</td>
</tr>
<tr>
<td>FinHER</td>
<td>Finland HERceptin</td>
</tr>
<tr>
<td>HERA</td>
<td>Herceptin adjuvant</td>
</tr>
<tr>
<td>NCCTG</td>
<td>North Central Cancer Treatment Group</td>
</tr>
<tr>
<td>Neo-ALTTO</td>
<td>Neoadjuvant Lapatinib and/or Trastuzumab Treatment Optimisation</td>
</tr>
<tr>
<td>NSABP</td>
<td>National Surgical Adjuvant Breast and Bowel Project</td>
</tr>
<tr>
<td>PHARE</td>
<td>Protocol of Herceptin Adjuvant with Reduced Exposure</td>
</tr>
</tbody>
</table>
and data from the FinHER (FINland HERceptinstudy (short 9-week course of trastuzumab) need to be taken into consideration.\textsuperscript{15} In addition, two other trials will investigate shorter trastuzumab therapy: the PHARE trial\textsuperscript{16} will compare 6 versus 12 months and SHORTHER\textsuperscript{17} will compare 3 versus 12 months. Moreover, although concomitant administration of trastuzumab with chemotherapy showed an improved DFS benefit compared with the sequential arm in the N9831 trial\textsuperscript{18} this schedule was associated with a potential increase of cardiac toxicity.

The role of lapatinib, a dual tyrosine kinase inhibitor (TKI) that targets both HER1 (epidermal growth factor receptor [EGFR]) and HER2, in the adjuvant treatment of HER2-positive EBC, either alone or in combination with or sequential to trastuzumab, has been investigated in the ALTTO trial. Although data are still pending, after a planned interim review of early results, the independent data monitoring committee has indicated that the lapatinib-alone arm is unlikely to meet the pre-specified criteria to demonstrate non-inferiority to trastuzumab alone with respect to DFS. Consequently, patients assigned to the lapatinib-alone arm of the trial were invited to discontinue lapatinib and discuss treatment options with their study physician. On the other hand, the remaining three arms of the trial were maintained as planned.

\subsection*{2.1.2 Neoadjuvant Treatment}

Even in the neoadjuvant setting, treatment with anti-HER2 agents was associated with improved results.

In a randomized trial in locally advanced and inflammatory HER2-positive breast cancer, the administration of 1 year of trastuzumab in neoadjuvant and adjuvant settings was associated with an improved pathological complete response (pCR) rate and event-free survival (HR = 0.59, 95\% CI 0.38, 0.90).\textsuperscript{19} In this trial, trastuzumab was administered concomitantly with anthracycline-containing chemotherapy.

In another small randomized trial, the concomitant administration of trastuzumab with preoperative chemotherapy resulted in improved response rates and DFS.\textsuperscript{20}

In both of these studies, trastuzumab was administered concomitantly with anthracycline- and taxane-based chemotherapy. Safety data show a favourable profile, with a low incidence of clinically significant cardiac adverse events.

Several preclinical and clinical data strongly suggest that combination of different HER2 target agents working with different mechanisms of action may improve the outcome in patients with HER2-positive disease, as trastuzumab and lapatinib have been clearly shown to be inadequate to inhibit the HER2 network completely when used as single agents.\textsuperscript{21-23} Recent data from the Neo-ALTTO trial (a neoadjuvant study that enrolled 450 patients with HER2-positive tumours >2 cm randomized to trastuzumab, lapatinib or the combination for 6 weeks, at which time paclitaxel was added to each of the arms for an additional 12 weeks before surgery) showed that pCR, defined as no invasive cancer in the breast or only ductal carcinoma \textit{in situ} in the breast specimen, was significantly higher in the combination arm (51.3\% in trastuzumab plus lapatinib vs 29.5\% in the trastuzumab and 24.7\% in the lapatinib arms, respectively).\textsuperscript{24} After surgery, all three arms will receive adjuvant chemotherapy with fluorouracil, epirubicin, cyclophosphamide (FEC) followed by the respective HER2 inhibitor either alone or in combination for 34 weeks. Consistent with this evidence, the adjuvant trial ALTTO is currently ongoing.

Trastuzumab has been shown to block ligand-independent association between HER2 and HER3, whereas pertuzumab, an antibody that recognizes an epitope in heterodimerization domain II of HER2, blocks ligand-induced HER2/HER3 dimerization.\textsuperscript{25} In trastuzumab-resistant xenografts, and in patients with HER2-positive breast cancer that has progressed on trastuzumab, only the combination of pertuzumab and trastuzumab, but not each antibody alone, exhibited clinical activity, stressing again the need to use different agents to completely inhibit HER2 pathway.\textsuperscript{26,27} Data from a recent clinical trial in patients with HER2-positive primary breast cancer (NeoSphere) further confirm this concept. In NeoSphere, the pCR rate was 45.8\% versus 29\% (p = 0.01) in patients treated with neoadjuvant docetaxel/
trastuzumab/pertuzumab versus docetaxel/trastuzumab, respectively, suggesting that both HER2 antibodies might be required to completely inhibit HER2/HER3 dimerization in situ, potentially explaining their clinical activity in combination.\[^{28}\] Another large phase III clinical trial, the Cleopatra study, randomized patients with HER2-positive MBC to trastuzumab and docetaxel with or without pertuzumab as first-line therapy using progression-free survival (PFS) as a primary endpoint.\[^{29}\] The results from this study were recently published\[^{29}\] and the addition of pertuzumab to trastuzumab and docetaxel revealed a statistically significant advantage in median PFS (18.5 months vs 12.4 months, HR 0.62; 95% CI 0.51, 0.75; \(p<0.001\)).

2.2 Advanced Disease

Trastuzumab has demonstrated therapeutic benefit in women with HER2-positive MBC, both in first-line and salvage settings. Initially, phase II clinical trials established its activity as monotherapy,\[^{30,31}\] but it was soon evident that a better performance could be obtained by combining trastuzumab with chemotherapy. Namely, a pivotal phase III study showed that the combination of trastuzumab and chemotherapy significantly prolonged time to progression (TTP) and OS compared with chemotherapy alone in patients with HER2-positive MBC.\[^{8}\] Similarly, in a randomized phase II trial, the addition of trastuzumab to docetaxel improved response rate, TTP and OS compared with docetaxel alone as first-line treatment of HER2-positive MBC.\[^{9}\]

Preclinical studies in HER2-amplified breast cancer cell lines demonstrated synergistic or additive interactions between trastuzumab and a variety of cytotoxic agents, prompting intense evaluation of trastuzumab with different types of chemotherapeutic agents within clinical trials.\[^{32}\] Taxanes (paclitaxel and docetaxel) with or without platinum salts, vinorelbine and capecitabine are all potential partners of trastuzumab in the first-line treatment of HER2-positive MBC.\[^{33}\] To date, no clear evidence exists that regimens in which trastuzumab is added to two different chemotherapeutic agents are superior to combinations\[^{34}\] in which trastuzumab is added to single-agent chemotherapy.

Based on the hypothesis that, under the selective pressure of continuous trastuzumab exposure, the antiproliferative action of the monoclonal antibody could be bypassed by breast cancer cells while its chemotherapy-sensitizing effect is maintained, the benefit of continuing anti-HER2 treatment beyond progression was examined in a phase III randomized trial that supported this strategy.\[^{35,36}\] Although prematurely closed, the study demonstrated that the combination of trastuzumab plus capecitabine resulted in a significant improvement in overall response and TTP compared with capecitabine alone in patients with HER2-positive breast cancer who experienced progression during trastuzumab treatment. Another RCT with a similar concept but examining a different population demonstrated the advantage of the switch to capecitabine plus lapatinib in patients who experienced progression after trastuzumab-based therapy.\[^{37,38}\]

In addition, recent data suggest that reintroduction or continuation of trastuzumab improves even the efficacy of lapatinib in patients with HER2-positive MBC who are heavily pretreated.\[^{23}\]

3. Potential Mechanisms of Resistance to Trastuzumab

3.1 Potential Mechanisms of Action of Trastuzumab

The exact antitumour mechanisms of action of trastuzumab are not completely understood.\[^{39}\] Nevertheless, it is clear that they follow the binding of the monoclonal antibody to the ECD of the HER2 receptor.\[^{40}\] Antibody-dependent cellular cytotoxicity (ADCC) mediated by CD56-positive natural killer cells has been proposed as contributing to the activity of trastuzumab in HER2-positive and perhaps also in HER2-negative tumours.\[^{41-43}\] In addition, trastuzumab binding to the HER2 receptor has been shown to inhibit its proteolytic shedding and, in turn, the formation of p95HER2, the truncated and constitutively active form of the receptor with kinase activity.\[^{44}\] Trastuzumab is also able to prevent
ligand-independent HER2 receptor dimerization. Among other potential mechanisms of action, it has been proposed that trastuzumab may determine internalization and degradation of HER2 by recruitment of c-Cbl, a ubiquitin-protein isopeptide ligase that causes rapid removal of HER2 from the cell surface. Overall, the antitumour effect of trastuzumab may be exerted through interference with downstream signalling pathways, inhibition of cell-cycle arrest and induction of apoptosis. In addition, HER2-positive tumours often show high intratumoral vascular endothelial growth factor (VEGF) expression, and trastuzumab could also work as an antiangiogenic agent. Of note, a study conducted in a xenograft model demonstrated trastuzumab-induced normalization and regression of the tumour vasculature. Finally, to explain the synergistic effect of trastuzumab with several chemotherapy agents, some studies have evaluated the role of trastuzumab in interfering with mechanisms of repair of treatment-induced DNA damage. Interestingly, it has been reported that trastuzumab is able to inhibit expression of p21/WAF1, a protein implicated in the cellular response to DNA damage, either at baseline or after exposure to cisplatin.

3.2 Cross-Talk with Heterologous Receptor Tyrosine Kinases and Amplification of Human Epidermal Growth Factor Signalling

A potential mechanism of trastuzumab resistance involves RTKs outside of the HER family modulating levels of the cyclin-dependent inhibitor (Cdk) kinase 1B (p27kip1), such as the insulin-like growth factor (IGF)-1 receptor (IGF-1R). For example, trastuzumab-induced growth inhibition was lost in breast cancer cells that overexpressed both IGF-1R and HER2. Indeed, the overexpression of IGF-1R or increased levels of IGF-1R/HER2 heterodimers potently activate PI3K and its downstream effector AKT, abrogating trastuzumab action when transfected into antibody-sensitive breast cancer cells. Furthermore, the reduction of IGF-1R signalling by recombinant IGF binding protein 3 when combined with trastuzumab resulted in the reduction of proliferation in trastuzumab-resistant cells. In a different trastuzumab-resistant cell model, the inhibition of IGF-1R expression with small interfering RNA (siRNA) or the inhibition of IGF-1R tyrosine kinase activity by NVP-AEW541 increased the antiproliferative activity of trastuzumab re-challenge.

In a neoadjuvant trial of chemotherapy plus trastuzumab, high levels of IGF-1R as measured by immunohistochemistry (IHC) correlated with a poor clinical response. However, data from another clinical trial suggest that the expression of IGF-1R alone per se does not predict trastuzumab resistance in patients with HER2-overexpressing breast cancer and that the activation of IGF-1R signalling may be more important than its expression for development of resistance to trastuzumab. Indeed, IGF-1 signalling resulted in elevated expression of the p27kip1 ubiquitin ligase SKP2, leading to decreased p27kip1 and loss of growth arrest in the presence of trastuzumab. Furthermore, cross-talk occurs between IGF-1R and HER2, and IGF-1R physically interacts with HER2 and induces activation of HER2 in trastuzumab-resistant, but not trastuzumab-sensitive, breast cancer cells. Given the reportedly complex interactions between the RTK ligands, receptors and signalling pathways, it is perhaps not surprising that HER2-overexpressing breast cancers have shown variable responses to trastuzumab.

Metabolic dysregulation, particularly increased glycolysis as a source of energy for cell survival, seems to play a role in carcinogenesis and could be associated with resistance to anticancer treatment. It has been demonstrated that HER2 activation leads to the upregulation of lactate dehydrogenase-A (LDH-A) through heat shock factor (HSF)-1 and the promotion of glycolysis and cell growth. Trastuzumab inhibits glycolysis and HSF1 and, consequently, reduces cell growth. Upregulation of HSF1 could be responsible for trastuzumab resistance and enhanced glycolysis despite antiHER2 treatment. In trastuzumab-resistant cells, the combination of trastuzumab and a glycolysis inhibitor (such as 2-deoxy-D-glucose or oxamate) restored cell inhibition. In addition, interaction between trastuzumab and oxamate was demonstrated to be synergistic in reducing
tumour growth.\textsuperscript{[56]} The combination of glycolysis inhibitors and anti-HER2 treatment deserves to be further investigated.

The RTK MET (HGF receptor) has also been implicated in trastuzumab resistance. HER2-overexpressing cells upregulate MET following exposure to trastuzumab. Further, activation of MET protects cells against trastuzumab by abrogating the induction of p27.\textsuperscript{[57]}

In a cohort of patients with HER2-positive breast cancers, overexpression of the EphA2 RTK was associated with reduced DFS and OS. Treatment of resistant cells with trastuzumab induced phosphorylation of the tyrosine kinase SRC and EphA2, resulting in the activation of PI3K/AKT and mitogen-activated protein kinase (MAPK) signalling pathways. Administration of a neutralizing EphA2 antibody restored sensitivity to trastuzumab in vivo.\textsuperscript{[58]} Finally, the receptor for erythropoietin (EpoR) is co-expressed in a proportion of cell lines and primary tumours that also harbour HER2 gene amplification. In those cells, treatment with recombinant human erythropoietin (rHuEPO) activates the JAK and SRC tyrosine kinase, leading to inactivation of the tumour suppressor PTEN and attenuation of the response to trastuzumab.\textsuperscript{[59]}

Other members of the HER-receptor network and their overexpression are thought to play a role in trastuzumab resistance. Coexpression of HER3 and HER2 is frequently observed in breast cancers and cell lines.\textsuperscript{[60]} HER2 requires HER3 to promote breast cancer cell proliferation.\textsuperscript{[61,62]} Elevated expression of HER3 and its association with HER2 promote mammary tumorigenesis in c-neu transgenic models.\textsuperscript{[63,64]} Exogenous ligands of the HER1 and HER3/4 co-receptors have been shown to provide rescue from the anti-proliferative effect of the antibody 4D5, which targets HER2.\textsuperscript{[65,66]} This is consistent with structural data using HER-receptor ectodomains, which show that trastuzumab is unable to block ligand-induced HER1/HER2 and HER2/HER3 heterodimers.\textsuperscript{[25,67]}

Recent studies reported that trastuzumab-resistant HER2-overexpressing BT-474 human breast cancer cells retained HER2 gene amplification and trastuzumab binding but exhibited higher levels of phosphorylated HER1 and HER3 and HER1/HER2 heterodimers as well as overexpression of HER1, transforming growth factor (TGF)-z, heparin-binding EGF-like growth factor (HB-EGF) and heregulin RNAs compared with the parental trastuzumab-sensitive cells, thus suggesting enhanced HER1- and HER3-mediated activation of HER2.\textsuperscript{[68]}

The dual HER1/HER2 TKI lapatinib and the HER2 antibody pertuzumab, which blocks HER2 heterodimerization with HER co-receptors,\textsuperscript{[69,70]} inhibited growth of the antibody-resistant cells further suggesting that, although resistant to trastuzumab, the cells were still dependent on HER2-dependent interactions with the HER receptor network.\textsuperscript{[68]}

In line with this report, the activation of TGF\textbeta\ receptor, a pathway amplified in metastatic mammary tumours, has been shown to induce phosphorylation of the sheddase tumour necrosis factor (TNF)-z-converting enzyme (TACE)/a disintegrin and metalloprotease-17 (ADAM17) resulting in increased secretion of TGFz, amphiregulin and heregulin. These changes are followed by enhanced coupling of the p85 subunit of PI3K and HER3, activation of PI3K/AKT and resistance to trastuzumab. Further, a gene signature induced by expression of a constitutively active, mutant type I TGF\textbeta receptor correlated with resistance to trastuzumab in a panel of HER2-positive breast cancer cells lines and with poor clinical outcome in patients with invasive breast cancer.\textsuperscript{[71]}

Considering the importance of both IGF-1R and HER3 in HER2-mediated breast cancer development, it is conceivable to predict that co-targeting IGF-1R and HER3 signalling may ultimately enhance the therapeutic efficacy of trastuzumab in HER2-overexpressing breast cancers. In this regard, Huang et al.\textsuperscript{[72]} recently reported enhanced activation of downstream signalling pathways emanating from the growth factor receptors HER2, HER3 and IGF-1R in trastuzumab-resistant breast cancer cells. In their study, interactions between IGF-1R and HER2 or HER3 occur exclusively in trastuzumab-resistant
cells, where enhanced HER2-HER3 interactions are also observed. Moreover, these three receptors form a heterotrimeric complex in resistant cells. HER3 or IGF-1R knockdown by short hairpin RNA-mediated strategies upregulates p27kip1, inactivates downstream receptor signalling and resensitizes trastuzumab-resistant cells.

On the basis of these results, trastuzumab resistance in breast cancer might be overcome by therapeutic strategies that jointly target HER2, HER3 and IGF-1R.

3.3 Amplification of the PI3K/AKT Pathway

Resistance to trastuzumab may occur as a result of aberrant activation of signalling pathways downstream of the receptor, such as PI3K/AKT. Molecular alterations involving this pathway are considered the most frequent in breast cancer, together encompassing over 30% of invasive tumours. Alterations in breast cancer resulting in hyperactivity of the PI3K pathway include gain-of-function mutations in PIK3CA (the gene encoding the PI3K catalytic subunit p110α),[73,74] mutations in AKT1,[75] amplifications of AKT2,[76] loss of the PTEN lipid phosphatase,[77,78] and loss of the tumour suppressor INPP4B (inositol polyphosphate 4-phosphatase type II).[79]

PIK3CA mutations in primary breast tumours have been associated with lymph node metastases, the presence of hormonal (estrogen and progesterone) receptors and HER2 overexpression.[80,81] It is generally accepted that anti-HER2 therapies should inhibit PI3K/AKT signalling downstream of the HER2 receptor in order to inhibit tumour growth.

Supporting data for a major role of the PI3K pathway comes from a large-scale RNA interference screen to discover genes involved in trastuzumab resistance in breast cancer.[82] Their finding that knockdown of PTEN in BT-474 cells decreases sensitivity to trastuzumab is consistent with earlier findings that demonstrated that PTEN loss is associated with resistance to trastuzumab-based therapy[83] and in agreement with the observation that, in antibody-sensitive cells, trastuzumab increases the phosphatase activity of PTEN via inhibition of SRC and SRC-mediated (inhibitory) phosphorylation of PTEN.[83] Importantly, the observation that, of the 8000 genes tested, only knockdown of PTEN conferred resistance to trastuzumab, suggests that the PTEN pathway plays a dominant role in trastuzumab resistance. In the same work, the authors also showed that oncogenic mutants of PIK3CA, activator of the same pathway and frequently mutated in breast cancer, also conferred resistance to trastuzumab in cell culture. Significant loss of PTEN expression is seen in some 20–25% of HER2-positive breast cancers.[80,84] Activating mutations in PIK3CA have also been found in approximately 25% of primary breast cancers and these occur almost exclusively in the PTEN-negative samples.[80] In patients, analyses of PTEN status or PIK3CA mutation status alone had only limited ability to predict prognosis after trastuzumab treatment.[82] However, combined analysis of PTEN status and PIK3CA status not only identified twice as many patients at increased risk for disease progression, but the combined analysis also reached statistical significance as a biomarker for prognosis after trastuzumab therapy. In other words, PIK3CA mutation status and PTEN expression level, reflecting pathway activation status and their assessment may be very useful in predicting trastuzumab resistance in HER2-amplified breast tumours.[82]

Supporting aberrant PI3K signalling and causality to drug resistance, in more recent preclinical studies, the addition of PI3K inhibitors to trastuzumab has inhibited growth of HER2-positive/PIK3CA mutant tumours resistant to anti-HER2 therapy.[85-87] Interestingly, inhibitors of the mammalian target of rapamycin (mTOR), a serine-threonine kinase downstream of PI3K, have shown activity after progression on trastuzumab. Dalenc et al.[88] recently reported a multicentre phase II study of 55 women with HER2-positive MBC whose tumours were resistant to trastuzumab and taxanes. Patients were treated with the mTOR inhibitor everolimus, paclitaxel and trastuzumab, exhibiting an impressive partial response rate of 19% and an overall clinical benefit rate of 81%.

In a pooled analysis of two phase I/II trials in 47 advanced HER2-positive MBC patients
progressing with trastuzumab, the combination of trastuzumab and everolimus led to a clinical benefit of 34% and a median PFS of 4.1 months.\textsuperscript{[89]} Notably, patients with documented tumour PTEN loss experienced shorter OS, even if PFS was not directly affected by PTEN status. Unfortunately, investigators were not able to find any predictive factor for benefit of the combination of trastuzumab and an mTOR inhibitor among several biomarkers examined (PIK3AC activation, PTEN loss, phosphorylated AKT, phosphorylated SRC, phosphorylated S6 kinase).

3.4 Alterations in Binding of Trastuzumab to HER2/Complete Loss of HER2 Expression

Inability or reduced capacity of trastuzumab binding to HER2 makes trastuzumab unable to interfere with the HER2 heterodimers containing HER1 or HER3. Recently, overexpression of the membrane-associated glycoprotein mucin-4 (MUC4) has been shown to directly interact with HER2, masking trastuzumab-binding epitopes in the HER2 receptor and resulting in acquired resistance.\textsuperscript{[90,91]}

Another potential mechanism of resistance is the accumulation of truncated forms of the HER2 receptor that lack the extracellular trastuzumab-binding domain.\textsuperscript{[92]} Amino terminally truncated carboxyl terminal fragments of HER2, collectively known as p95HER2 or C-terminal fragments, are frequently found in HER2-expressing breast cancer cell lines and tumours.\textsuperscript{[93]} These fragments arise through the proteolytic shedding of the extracellular domain of full-length HER2\textsuperscript{[93-96]} or by alternative initiation of translation from two methionine residues (611 and 687) that are located before and after the transmembrane domain, respectively.\textsuperscript{[96]} The biological function of p95HER2 has not been fully characterized, although overexpression of p95HER2 has been shown to lead to growth of tumour xenografts in nude mice.\textsuperscript{[96]} The p95HER2 protein has kinase activity, and this activity is required for tumour growth;\textsuperscript{[95]} however, the mechanisms involved and their possible relationship with those used by full-length HER2 are unknown. The p95HER2 is expressed in up to 30% of HER2-positive breast cancers and is associated with increased nodal metastasis and shorter DFS when compared with patients who overexpress full-length HER2.\textsuperscript{[93,97]} These truncated receptor fragments retain kinase activity and promote mammary tumour progression and metastasis even more aggressively than full-length HER2.\textsuperscript{[98]} Because p95HER2 lacks the extracellular trastuzumab binding domain, these receptors are not inhibited by this antibody. It has been recently shown that tumour xenografts expressing p95HER2 are refractory to the inhibitory effects of trastuzumab.\textsuperscript{[96]} On the other hand, as p95HER2 retains its kinase activity, treatment of p95HER2-expressing cells with lapatinib inhibited p95HER2 phosphorylation, reduced downstream phosphorylation of AKT and MAPK and inhibited cell growth in MCF-7p95HER2 clones.\textsuperscript{[92]} Importantly, there was an association between p95HER2 expression and lack of clinical response to trastuzumab in HER2-amplified breast cancer patients.\textsuperscript{[99]} Analysis of a cohort of patients with HER2-positive MBC treated with trastuzumab and chemotherapy showed a very low response rate in tumours with cytosolic p95HER2 compared with those without.\textsuperscript{[92]} Lapatinib has been shown to inhibit the catalytic activity of p95HER2. Therefore, patients with p95HER2-positive breast cancers treated with lapatinib alone or in combination with capecitabine exhibited a similar DFS and overall response rate compared with p95HER2-negative tumours, suggesting a clinical setting where a HER2 TKI might be advantageous over trastuzumab.\textsuperscript{[99]}

Finally, an oncogenic splice isoform with an in-frame deletion of exon 16 (HER2Δ16) is found in some HER2-overexpressing breast cancer cell lines and primary breast cancers.\textsuperscript{[100,101]} Loss of exon 16 results in a constitutively dimerized and active HER2 receptor, enhanced SRC activity and accelerated transformation. Cells expressing HER2Δ16 are resistant to trastuzumab; this resistance is abrogated by co-treatment with SRC inhibitors.\textsuperscript{[102]} It has not been shown yet whether HER2Δ16 is a mechanism of resistance to trastuzumab in patients with HER2-positive tumours.
Another potential mechanism of acquired resistance for trastuzumab is the loss of HER2 expression during or after trastuzumab therapy. Mittendorf et al.\textsuperscript{[103]} confirmed that patients with HER2-overexpressing breast cancer treated in the neoadjuvant setting with trastuzumab-based systemic therapy achieve a high rate (about 50\%) of pCR. Importantly, in patients not achieving a pCR and with significant residual disease, fluorescence in situ hybridization (FISH) showed that the tumours from one-third of these patients no longer had amplification of the HER2 gene. Those patients with tumours that were no longer HER2 gene amplified had a significantly worse recurrence-free survival than those with tumours that retained HER2 gene amplification. In a study conducted to evaluate changes in HER2 status in metastatic lesions of patients previously treated with trastuzumab, Pectasides et al.\textsuperscript{[104]} showed that 37\% of patients no longer had HER2 expression/gene amplification, and these patients had significantly shorter time to tumour progression than the group who remained HER2-positive. Consistent with these findings, Hurley et al.\textsuperscript{[105]} showed that 43\% of tumours that had HER2 gene amplification by FISH before treatment with neoadjuvant trastuzumab, docetaxel and cisplatin became FISH-negative after therapy. Taken together, these data suggest that residual tumour identified at the time of surgery in patients receiving trastuzumab-based neoadjuvant therapy should be reassessed for HER2 status, as HER2 status may change over time as resistance arises and that novel adjuvant therapy strategies need to be studied in this population.

4. Potential Mechanisms of Resistance to Tyrosine Kinase Inhibitors

An alternative approach to target the HER2 and HER family receptor pathways is the use of ATP-competitive, small-molecule TKIs. The dual HER1/HER2 TKI lapatinib is active as first-line monotherapy in patients with HER2-positive MBC and, in combination with chemotherapy, improves PFS compared with chemotherapy alone.\textsuperscript{[106]} Like lapatinib, the HER1/HER2 dual TKI neratinib has shown clinical activity in patients with HER2-positive MBC who have progressed on trastuzumab.\textsuperscript{[107]} In a phase II trial, in HER2-positive MBC patients pre-treated or not with trastuzumab, neratinib demonstrated an objective response rate of 24\% (95\% CI 14, 36) and 56\% (95\% CI 43, 69), and a median PFS of 22.3 and 39.6 weeks, respectively. The most reported grade 3/4 adverse event was diarrhoea, which occurred more frequently in trastuzumab-pretreated patients (30\% vs 13\%).\textsuperscript{[108]} Currently, neratinib is under investigation in a phase III trial in HER2-positive EBC (after completion of trastuzumab: 1 year of neratinib vs placebo).

As with trastuzumab, it is generally accepted that in order to exert an antitumour effect in HER2-positive cancers, treatment with TKIs should inhibit the PI3K/AKT pathway.\textsuperscript{[107]} Proposed mechanisms of resistance to lapatinib involve recovery through derepression and/or activation of compensatory survival pathways. For example, in HER2-overexpressing BT474 cells selected for acquired resistance to lapatinib, the resistant cells continued to show inhibition of HER2, HER3, MAPK and AKT phosphorylation upon treatment with lapatinib. In these cells, inhibition of AKT with lapatinib resulted in derepression of the transcription factor FOXO3a thus leading to increased estrogen receptor (ER) transcription and ER signalling.\textsuperscript{[109,110]} Cotreatment with lapatinib and the ER downregulator fulvestrant prevented the outgrowth of drug-resistant cells. Further, lapatinib was shown to induce ER signalling in tumour biopsies from patients with HER2-positive/ER-positive but not HER2-positive/ER-negative breast cancers. The same group also found calcium-dependent increased levels of phosphorylated RelA, the prosurvival subunit of nuclear factor (NF)-kB, upon lapatinib treatment of HER2-positive breast cancer cell lines.\textsuperscript{[111]} Using either siRNA constructs targeting RelA or an intracellular calcium chelator enhanced the apoptotic effects of lapatinib, suggesting a possible role for RelA in adaptation to the HER2 TKI.

Using HER2-positive cells selected in culture, another study identified overexpression of AXL as a mechanism of resistance to lapatinib.\textsuperscript{[112]}

Adis © 2012 Springer International Publishing AG. All rights reserved.
AXL is an RTK with a kinase domain closely resembling MET and an extracellular domain resembling neural cell adhesion molecules. BT474 cells rendered drug resistant by chronic exposure to lapatinib exhibited increased expression and activation of AXL. GSK1363089 (foretinib), a multikinase inhibitor of AXL, MET and VEGFR, restored lapatinib and trastuzumab sensitivity in the AXL-overexpressing, drug-resistant cells and it is now being tested in phase II clinical trials. Other studies have shown upregulation of HER3 transcription and protein levels and recovery of HER3 phosphorylation after short-term inhibition of HER2 with the TKIs gefitinib and lapatinib. In one study, HER3-PI3K-AKT activity was completely inhibited by higher, pulsatile doses of lapatinib both in vitro and in vivo.
As with trastuzumab, activating PIK3CA mutations, loss of PTEN and alternative signalling pathways that activate PI3K-AKT are reported mechanisms for escape from lapatinib. Using a large-scale loss-of-function short hairpin RNA screen to identify novel modulators of resistance to lapatinib, Eichhorn et al.\textsuperscript{[86]} identified the PTEN as a gene whose loss reduced the sen-
sensitivity to the TKI both in vitro and in vivo. In addition, two dominant activating mutations in \textit{PIK3CA} (E545K and H1047R), which are prevalent in breast cancer, also conferred resistance to lapatinib. These authors also showed that the resistance to lapatinib induced by the PI3K mutants can be abrogated through the use of BEZ235, a dual inhibitor of PI3K/mTOR currently tested in phase I/II clinical trials.


Current clinical data support that trastuzumab-resistant tumours, even in advanced stages, continue to be dependent on the HER2 pathways to survive.\[37,106,108,116\] Indeed, there is a plethora of agents that either target HER2 by different mechanisms or inhibit molecules implicated in the development of resistance to HER2 target agents (figures 1 and 2). These different agents are at different stages of clinical development, as summarized by tables II–IV.

Recent data from two neoadjuvant studies (Neo-ALTTO\[24\] and the NeoSphere trial\[28\]) and one study in the MBC setting (Cleopatra\[29\]) clearly show that different HER2 target agents working with different mechanisms of action may improve the outcome in patients with HER2-positive breast cancer. Based on these encouraging findings, two further different HER3 monoclonal antibodies, U3-1287 and MM-121, are completing phase I testing.\[82,117\]

Trastuzumab emtansine, an antibody-drug conjugate of trastuzumab covalently bonded to three molecules of the microtubule polymerization inhibitor derivative of maytansine, DM1 is a novel HER2 inhibitor currently being tested in the clinical setting.\[118\] Trastuzumab emtansine binds to HER2 with an affinity similar to that of trastuzumab and, like trastuzumab, has the ability to inhibit signalling and engage immune effectors that mediate ADCC. Interestingly, preclinical study shows that trastuzumab emtansine is also active against lapatinib-resistant xenografts.\[119\] In patients with HER2-positive MBC who had progressed after trastuzumab and lapatinib, phase I–II studies of trastuzumab emtansine demonstrated remarkable clinical response rates, with only a mild and reversible toxicity.\[120,121\]

Recently, results of a randomized, multicentre,
open-label phase II study of trastuzumab emtansine versus trastuzumab plus docetaxel in previously untreated HER2-positive MBC were presented.\[122\] A significantly longer PFS was observed in the trastuzumab emtansine arm (14.2 months vs 9.2 months, HR = 0.59, 95% CI 0.36, 0.96; p = 0.03). Interestingly, a durable response for trastuzumab emtansine has been documented by the observation that the median of duration of response has not been reached. In addition, a lower rate of grade ≥3 adverse events (46.4% vs 89.4%) occurred with trastuzumab emtansine. Based on these encouraging findings, trastuzumab emtansine is now being evaluated in two large phase III randomized studies: one is comparing trastuzumab emtansine versus trastuzumab emtansine plus pertuzumab versus the standard of trastuzumab plus a taxane in patients with HER2-positive MBC previously untreated in the metastatic setting; the other is comparing trastuzumab emtansine versus the standard of lapatinib and capecitabine in similar patients but who have previously received trastuzumab.\[120,121\]

Heat shock protein (HSP)-90 is a ubiquitous chaperon protein, commonly overexpressed in cancer and acting by promoting the proper fold-

| Table III. Small molecules targeting HER family receptors or their downstream signaling currently in clinical development or already available in clinical practice |
|---|---|---|---|
| **Drugs** | **Features** | **Mechanism** | **Ongoing trials** |
| Lapatinib | Reversible TKI, small molecule | Reversible, competitive binding to ATP pocket in HER2 and EGFR | NCT00374322 (TEACH), NCT00829166 (EMILIA), NCT00567554 (GEPA- QUINTO), NCT00490139 (ALTTO), NCT00553358 (Neo-ALTTO), NCT00486668 (NSABP B-41), NCT00770809 (CALGB40601) |
| Neratinib | Irreversible TKI, small molecule | Covalent binding to ATP pocket in HER2 and EGFR | NCT00878709 (ExteNET), NCT00915018 (NEFERTT) |
| Everolimus, temsirolimus, ridaforolimus | mTOR inhibitors | Non catalytic inhibitors of TORC1 | NCT00876395 (BOLELO-1), NCT01007942 (BOLELO-3) |
| OSI-027 | Small molecule | Catalytic inhibitor of TORC1 and TORC2 | |
| MK-2206, AZDS363 | Small molecule | AllostERIC or catalytic inhibitors of AKT1/2 | NCT00963547, NCT01319539 |
| Linsitinib | Small molecule | Inhibition of Ins R and IGF-1R tyrosine kinases | NCT01205685 |
| BKM120, pictrelisib, SAR-24508 | Small molecule | PI3K inhibitors, ATP competitive inhibitors of p110 | NCT01300962 (LCCC 1024), NCT00928330, NCT01132664 |
| SAR-245409 | Kinase inhibitors | Inhibits PI3K and mTOR kinase activity by binding to the ATP-binding cleft of these enzymes | NCT01471847 |
| HSP90 inhibitors | | Induce destabilization and eventual degradation of HSP90 client proteins such as HER2 and other signal transducers | NCT00627627 |
| MET TKI, SRC, TGFβ inhibitors | Small molecules | Hepatocyte growth factor/scatter factor inhibition; c-SRC kinase inhibitor; TGFβ type I receptor kinase inhibitor | |
| MMP inhibitors | | Prevents ectodomain shedding (HER2 cleavage) | |
ing of protein. Inhibitors of HSP90 are expected to induce proteasome-mediated degradation of HSP client proteins, including proteins involved in cell growth signalling such as HER2. Tanespimycin is a geldanamycin derivative that has shown antitumour activity in several murine models (BRAF mutant melanoma, androgen receptor-dependent prostate cancer, mutant HER1 lung cancer and HER2-overexpressing breast cancer). Tanespimycin has been shown to degrade p95HER2 in trastuzumab-resistant breast cancer, with reduction of the expression of either full-length HER2 and inhibition of AKT activation, induction of apoptosis and growth control. Tanespimycin showed activity in a phase II trial among HER2-positive trastuzumab-resistant MBC. Patients received intravenous tanevimycin 450 mg/sqm weekly in combination with the conventional dose of trastuzumab; they achieved a response rate of 22%, with a clinical benefit of 59%. However, further clinical development of this drug has been discontinued.

In preclinical studies, the addition of PI3K pathway antagonists to trastuzumab or lapatinib inhibited growth of HER2-positive tumours resistant to anti-HER2 therapy. Several of these antagonists are now in clinical development. Interestingly, inhibitors of mTOR, a serine-threonine kinase downstream PI3K, have shown activity after progression on trastuzumab. Dalenc et al. recent-
ly reported a multicentre phase II study of 55 women with HER2-positive MBC whose tumours were resistant to trastuzumab and taxanes. Patients treated with the mTOR inhibitor everolimus, paclitaxel and trastuzumab exhibited an impressive partial response rate of 19% and an overall clinical benefit rate of 81%, confirming, in a clinical setting, the relevance of inhibiting the PI3K pathways to block tumour growth in these patients.

6. Future Directions

At this time, only trastuzumab and lapatinib are approved by the US FDA for the treatment of patients with HER2-overexpressing breast cancer. Though these treatments have already proven to be very active in patients with HER2-positive breast cancer, de novo and acquired resistance still represent a major problem limiting their efficacy. Furthermore, the lack of clinical validated biomarkers predictive for anti-HER2 therapy resistance makes it impossible for the physician to select the best therapeutic strategy for a specific patient. There are a plethora of agents that either target HER2 by different mechanisms or inhibit the mechanisms of resistance summarized in this article. Potential strategies to overcome resistance to anti-HER2 treatment have been investigated and several drugs are currently in different phases of clinical development. Available clinical data strongly suggest that the increasing use of dual HER2 blockade with trastuzumab and lapatinib as well as the development of novel anti-HER2 combinations will markedly limit or eventually abrogate acquired resistance to primary anti-HER2 therapy.

Profiling of HER2-positive primary breast cancer and metastatic recurrences following anti-HER2 therapy will be critical to help to clarify, in the future, which of the molecular mechanisms of resistance are more relevant in the clinic and to identify potential therapeutic targets and biomarkers able to predict treatment efficacy.

Acknowledgements

The authors thank Dr Roberta Sottile for her assistance in preparing the manuscript.

Dr Fabio Puglisi has received consulting fees or honorarium from Roche and GlaxoSmithKline. Dr Grazia Arpino has received consulting fees or honorarium from Roche and GlaxoSmithKline. All other authors have no conflicts of interest that are directly relevant to the content of this article.

References

71. Wang SE, Xian B, Guix M, et al. Transforming growth factor beta engages TACE and ErbB3 to activate phos-
phatidylinositol-3 kinase/Akt in ErbB2-overexpressing breast cancer and desensitizes cells to trastuzumab. Mol Cell Biol 2008; 28: 5605-20


110. Guo SQ, Sonenshein GE. Forkhead box transcription factor FOXO3a regulates estrogen receptor alpha expression and is repressed by the Her-2/neu/phosphatidylinositol 3-kinase/Akt signaling pathway. Mol Cell Biol 2004; 24: 8681-90


Correspondence: Dr Fabio Puglisi, MD, PhD, University Hospital of Udine, Piazzale SM Misericordia, 15, I-33100 Udine, Italy.
E-mail: fabio.puglisi@uniud.it