

Resistance to Trastuzumab in Breast Cancer

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Abstract HER2 is a transmembrane oncoprotein encoded by the *HER2/neu* gene and is overexpressed in approximately 20 to 25% of invasive breast cancers. It can be therapeutically targeted by trastuzumab, a humanized IgG1 *kappa* light chain monoclonal antibody. Although trastuzumab is currently considered one of the most effective treatments in oncology, a significant number of patients with HER2-overexpressing breast cancer do not benefit from it. Understanding the mechanisms of action and resistance to trastuzumab is therefore crucial for the development of new therapeutic strategies. This review discusses proposed trastuzumab mode of action as well as proposed mechanisms for resistance. Mechanisms for resistance are grouped into four main categories: (1) obstacles preventing trastuzumab binding to HER2; (2) upregulation of HER2 downstream signaling pathways; (3) signaling through alternate pathways; and (4) failure to trigger an immune-mediated mechanism to destroy tumor cells. These potential mechanisms through which trastuzumab resistance may arise have been used as a guide to develop drugs, presently in clinical trials, to overcome resistance. The mechanisms conferring trastuzumab resistance, when completely understood, will provide insight on how best to treat HER2-overexpressing breast cancer. The understanding of each mechanism of resistance is therefore critical for the educated development of strategies to overcome it, as well as for the development of tools that would allow definitive and efficient patient selection for each therapy. (Clin Cancer Res 2009;15(24):7479–91)

In the past four decades the development of strategies for the treatment of breast cancer has focused on understanding the expression, regulation, and function of critical signaling pathways involved in cancer initiation and progression. This process allowed the identification of breast cancer subsets with distinct biology (1–4), as well as the development of targeted therapies. Notable examples are the successful use of hormonal therapy for women with hormone-sensitive tumors (5), and the use of anti-human epidermal growth factor receptor 2 (HER2) therapy for women with HER2-overexpressing tumors (6).

HER2 is a 185-kDa transmembrane oncoprotein (p185) encoded by the *HER2/neu* gene and overexpressed in approximately 20 to 25% of invasive breast cancers (7, 8). *HER2/neu* was initially identified in a rat glioblastoma model (7, 9), and then linked to an aggressive biological behavior in breast cancer,

which translated into shorter disease-free interval and overall survival in patients with early and advanced disease (10).

HER2, also known as ErbB2, is a tyrosine kinase (TK) receptor. It is a member of the HER (or ErbB) growth factor receptor family. This family of receptors comprise four distinct receptors, the epidermal growth factor receptor (EGFR) or ErbB1, HER2 (or ErbB2), HER3 (or ErbB3), and HER4 (or ErbB4; ref. 8). Homo- or heterodimerization of these receptors results in phosphorylation of residues from the intracellular domain of the receptor. This results in the recruitment of signaling molecules from the cytoplasm and initiation of several signaling pathways. The most studied HER2 downstream signaling pathways are the RAS/Raf/Mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K)/Akt cascades. Figure 1 illustrates some intracellular effects of homo- and heterodimerization of HER2. HER2 dimerization triggers a number of processes in the cell, culminating in increased cell motility, survival and proliferation, as well as resistance to apoptosis (11).

One of the most successful strategies in the development of targeted therapy in oncology has involved the production of monoclonal antibodies (mAb) directed against epitopes present on tumor cells. Likewise, antibody-based therapy targeting HER2 is based on the development of mAbs against epitopes present in the HER2 extracellular domain. Upon binding to their cognate epitopes, these antibodies exert their antitumor effects by a variety of proposed mechanisms. The clinical use of anti-HER2 extracellular domain mAbs contrasts with another therapeutic approach involving TK inhibitors, small molecules that target the ErbB receptor kinases from intracellular domains to prevent downstream signaling through the receptor.

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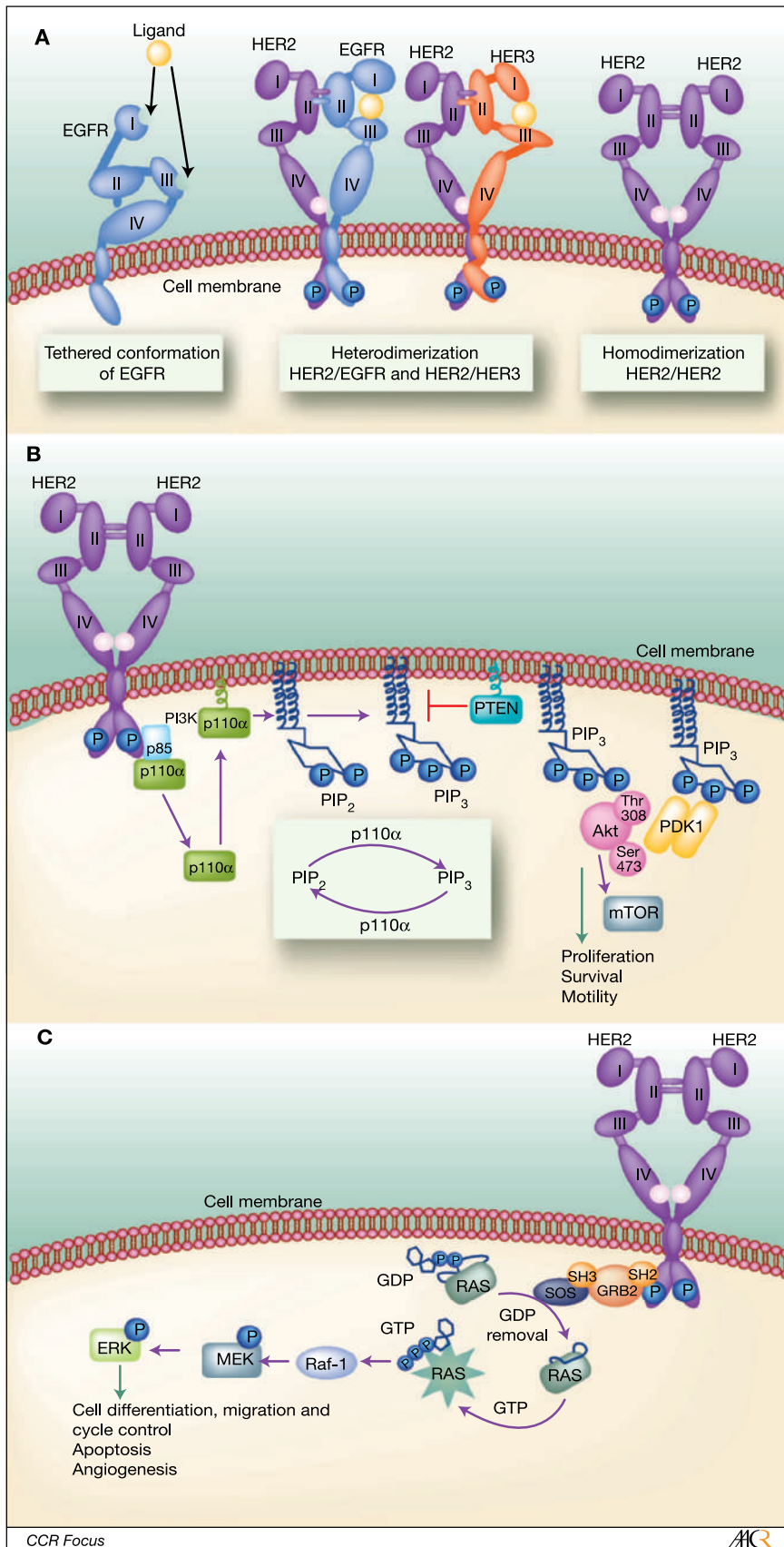


Fig. 1. HER2 activation. **A**, receptor dimerization is required for HER2 function (8). In the absence of a ligand, EGFR (represented in blue), HER3, and HER4 assume a tethered conformation. In tethered receptors the dimerization site from extracellular domain II is hidden by intramolecular interactions between domains II and IV. Growth factors alter the conformation of these receptors by binding simultaneously to two sites on extracellular domain domains: I and III (122). HER2 (represented in purple) occurs in an open position and is naturally ready for dimerization. Although no ligand has been identified for HER2 (123), the receptor may become activated by homodimerization (HER2/HER2 pair) or heterodimerization (represented in figure by EGFR/HER2 and HER2/HER3 dimers). The role of HER4 in oncogenesis is less defined (HER2/HER4 dimer not represented). The activation of the HER2 receptor triggers a number of downstream signaling steps through cytoplasm and nucleus, culminating with increased cell growth, survival, and motility (for reviews see refs. 68, 124–127). **B**, activation of PI3K/Akt pathway is one of the most studied processes involved with HER2 activation. PI3K is composed of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit, stably bound to each other and inactive in quiescent cells. Upon activation by the TKs from HER2, p85 binding to the receptor TKs relieves p110 α catalytic unit to relocate at the plasma membrane. There p110 α phosphorylates and converts phosphatidylinositol (4,5)-bisphosphate (PIP₂) into phosphatidylinositol (3,4,5)-triphosphate (PIP₃). PIP₃ acts as a docking site for pleckstrin homology (PH)-containing proteins, such as Akt and phosphatidylinositol-dependent kinase 1 (PDK1). At the membrane, Akt bound to PIP₃ becomes phosphorylated at threonine 308 and serine 473. PDK1 also contributes with Akt activation. Activated Akt activates mTOR and has several other intracellular effects, including interaction with transcription factors, metabolic pathways, apoptosis, and angiogenesis, resulting in cell proliferation, invasion, and survival (128). PIP₃ in turn is dephosphorylated back to PIP₂ by PTEN. PTEN is therefore a negative regulator of PI3K/Akt signaling and functions as a tumor suppressor. **C**, the RAS/Raf/MAPK signaling cascade is also triggered by HER2 activation. Growth Factor Receptor-Bound Protein 2 (GRB2) is an adaptor protein that contains one Src Homology 2 (SH2) domain, which recognizes the tyrosine-phosphorylated sites on the activated receptor for binding. GRB2 binds to the guanine nucleotide exchange factor Son of Sevenless (SOS) by one of its SH3 domains. When the GRB2/SOS complex docks to the activated receptor, SOS becomes activated and removes guanosine diphosphate (GDP) from inactive RAS. Free RAS can then bind guanosine-5'-triphosphate (GTP) and become active. RAS/GTP binds efficiently to Raf-1 (MAP3K), which becomes activated. Raf-1 can then activate MEK1 (MAP2K1) and MEK2 (MAP2K2), which are essential signaling nodes downstream RAS and Raf-1. MEK phosphorylates and activates the extracellular signal-regulated kinase (ERK) 1 and ERK2. Activation of ERK results in a broad spectrum of effects relevant to the cell physiology, including cell cycle control, differentiation, and migration, as well as apoptosis and angiogenesis.

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Trastuzumab (Herceptin) is a humanized IgG1 *kappa* light chain mAb in which the complementary-determining regions (CDR) of a HER2-specific mouse mAb were joined to human antibody framework regions through genetic engineering (12, 13). Trastuzumab has been approved by the U.S. Food and Drug Administration (FDA) for the treatment of HER2-overexpressing breast cancer in adjuvant and metastatic settings (6, 14–16). Nevertheless, a significant number of patients with HER2-overexpressing breast cancer will be initially or eventually resistant to anti-HER2-based therapy with trastuzumab (14, 17). Understanding the mechanisms of resistance to trastuzumab is therefore crucial for the development of new therapeutic strategies.

This *CCR Focus* will discuss targeted therapy resistance in different settings, including the treatment of non-small cell lung cancer (18), colorectal cancer (19), gastrointestinal stromal tu-

mors (20), chronic myeloid leukemia (21), and here, trastuzumab resistance in HER2-positive breast cancer. Resistance to anti-VEGF will also be discussed (22).

Trastuzumab

Trastuzumab complementary-determining region amino acids complement and bind to amino acids present on domain IV of the HER2 ectodomain (13, 23). The mechanism of action of trastuzumab is not fully understood and it is likely multifaceted. Being an IgG1, its proposed functions may be divided into those mediated by Fab (fragment, antigen binding) or Fc (fragment, crystallizable) regions, as represented in Fig. 2. The Fab contains the antigen-binding sites of the antibody, whereas the Fc contains the binding sites for Fc receptors present on immune

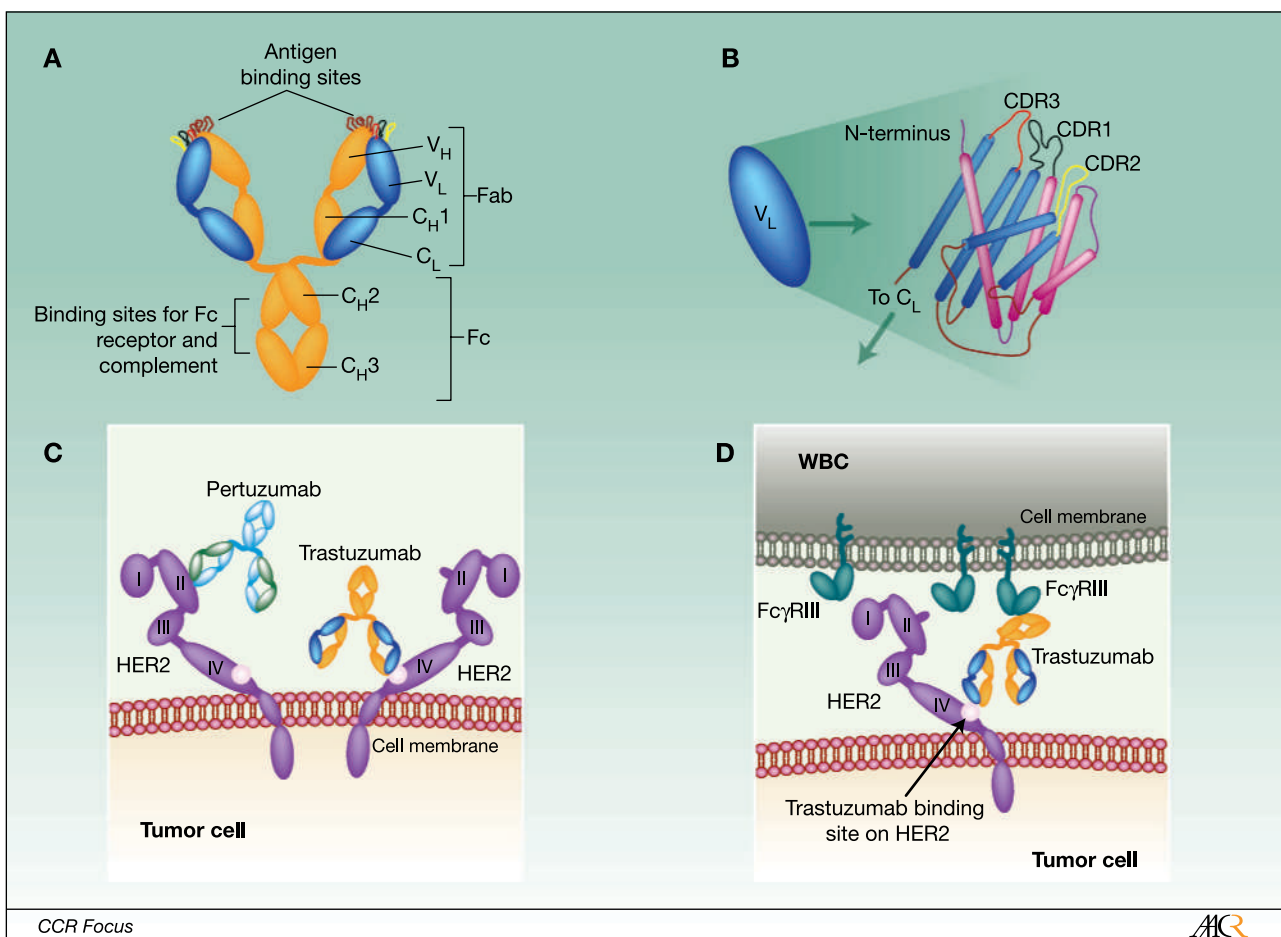


Fig. 2. Trastuzumab schematic structure. The structure of HER2 ectodomain in complex with trastuzumab Fab was described by Cho et al. (23). **A**, schematic of trastuzumab (IgG1 *kappa*). Brackets indicate the Fab and the Fc portions of IgG1. C_H1 to C_H3 indicate the heavy chain constant domains 1 to 3, whereas C_L indicates light chain constant domain. V_H and V_L denote variable heavy chain and variable light chain respectively. **B**, structural model of human IgG1 V_L adapted from Edmundson et al. (129). Complementarity-determining regions 1 to 3 represented in black, yellow, and red are also known as hypervariable regions. Complementarity-determining regions from variable heavy chain and from variable light chains are aligned and form a surface that complements the tridimensional antigen structure. The two sets of six complementarity-determining region loops in the antigen-binding sites are the only murine components of a humanized antibody such as trastuzumab. **C**, trastuzumab Fab-related function results from its binding to domain IV of HER2. HER2 indicates the human EGFR 2 (in purple). Pertuzumab, another anti-HER2 humanized mAb, binds to an epitope present on domain II of HER2. **D**, trastuzumab Fc-related functions result from the binding of its Fc portion to other cells that express Fc receptors, such as immune cells, hepatocytes, and endothelial cells. The Fc region of trastuzumab can bind to Fcγ receptor III (RIII) present on the surface of effector cells from the immune system and trigger tumor cell death via ADCC (29). WBC indicates white blood cell.

cells, platelets, hepatocytes, and endothelial cells (24, 25). The Fab portion is responsible for most of the studied pharmacodynamics effects of trastuzumab (26–28), whereas its Fc is relevant for both pharmacodynamics' (29, 30) and pharmacokinetics' (31–33) properties.

Trastuzumab is used in clinical doses considered capable of producing saturation of the receptor. When the conventional 4 mg/kg loading dose followed by 2 mg/kg weekly doses were given to 22 patients in a phase I trial (34), the mean maximum concentration of trastuzumab in plasma was approximately 70 µg/mL (95% confidence interval 64.7–79.1).

Trastuzumab Fab-related functions. Trastuzumab binds with high affinity to HER2 extracellular domain, producing a cytostatic effect that is associated with G1 arrest via upregulation of the cyclin-dependent kinase (Cdk) inhibitor p27 (28, 35–38). It blocks intracellular signaling via the PI3K/Akt pathway (28) and inhibits PI3K signaling by increasing the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) membrane localization and phosphatase activity (39).

In preclinical models the recruitment of HER3 to HER2 is critical for maintaining cell proliferation of HER2-overexpressing cell lines (40), and depends on the presence of a HER3 ligand (41). When ligand is present, trastuzumab may disrupt the interaction between EGFR and HER2, but it does not interfere with the interaction of HER2/HER3 (42). When HER2-amplified cells are cultured in the absence of ligand, trastuzumab leads to downregulation of proximal and distal Akt signaling regardless of the presence of HER3 (26, 43).

HER2 may undergo proteolytic cleavage that results in shedding of the extracellular domain and production of a truncated and phosphorylated (active) membrane-bound fragment, p95HER2. Trastuzumab inhibits HER2 cleavage and the generation of the active truncated p95HER2 *in vitro*, probably blocking the cleavage site of the receptor (27, 44, 45).

Trastuzumab modulates the effects of different pro- and anti-angiogenic factors as well, inducing normalization and regression of the vasculature in an animal model of HER2-overexpressing tumors (46). This includes inhibition of the production of the vascular endothelial growth factor (VEGF; refs. 36, 47).

Downregulation of HER2 through endocytosis has also been proposed as a mechanism of HER2 action, however, the available preclinical data are conflicting. Hommelgaard and colleagues (48) described the preferential association of HER2 with plasma membrane protrusions in the SKBR3 breast cancer cell line, which makes HER2 an internalization-resistant receptor. On the other hand, Yarden and colleagues (49, 50) proposed a mechanism by which antibodies may form large antigen-antibody lattices at the cell surface, which then collapse into the cytoplasm and undergo degradation in lysosomes. In this case aggregation and internalization would be increased with the concomitant use of antibodies targeting different epitopes on HER2.

Trastuzumab Fc-related functions. The importance of antibody-dependent cellular cytotoxicity (ADCC) function and of an operational Fc receptor for trastuzumab antitumoral effect was shown in several xenograft models (29, 51, 52). Gennari and colleagues (53) studied 11 patients treated with trastuzumab only in the neoadjuvant setting for 4 weeks, revealing high

levels of trastuzumab in patient serum, and saturating concentrations of trastuzumab in tumor tissue. Moreover, an infiltration by lymphoid cells was observed in all cases, and among patients presenting clinical responses, a higher capability to mediate *in vitro* ADCC was present. In addition, Musolino and colleagues (30) showed that certain FcγR polymorphisms in patients treated with trastuzumab are significantly associated with ADCC capacity in addition to such clinical endpoints as tumor response rates and progression-free survival.

The clinical importance of complement activation mediated by trastuzumab Fc is less clear. Trastuzumab has been shown to fix complement and to cause destruction of the HER2-positive cell line BT474 *in vitro* (52). On the other hand, in a model of glioblastoma multiforme treated with trastuzumab, complement-dependent cellular cytotoxicity (CDCC or simply CDC) was inefficient, possibly due to a strong expression of complement inhibitory factors (CD55 and CD59) on the HER2-positive glioblastoma cell lines used (A172 and U251MG; ref. 54).

Apart from the Fc-mediated cytotoxicity, the Fc portion of human IgG1 such as trastuzumab is important for maintaining the serum levels of the antibody. Intact IgG has been long recognized as more stable in serum and having longer half-life than Fab fragments (55, 56). Intact human IgG1 Fc binds to FcRn receptors on endothelial cells and on phagocytes, becomes internalized and recycled back to the blood stream to enhance its half-life within the body (25, 32, 57). This property of intact IgG1 molecules is critical for their stability and persistence in the circulation, and relevant to the process of the therapeutic mAb design (33, 58). Using a humanized FcRn xenograft model, Petkova and colleagues (59) evaluated the *in vivo* half-life of trastuzumab and of several trastuzumab Fc mutants, demonstrating shorter half-life of the mutant antibody containing a critical substitution of isoleucine at position 253 to alanine when compared with wild-type trastuzumab, as predicted by the mutant's failure to bind human FcRn (60). In a population pharmacokinetics study evaluating data from 476 patients from phase I, II, and III studies (61), a significant interpatient variation in trastuzumab clearance was found. Trastuzumab terminal half-life was estimated at 28.5 days (similar to that of endogenous IgG1), but the clinical pharmacokinetics of trastuzumab according to FcRn polymorphisms has not been described.

Mechanisms of Resistance to Trastuzumab

The most intensively studied general mechanisms of trastuzumab resistance are: (1) obstacles for trastuzumab binding to HER2; (2) upregulation of HER2 downstream signaling pathways; (3) signaling through alternate pathways; and (4) failure to trigger immune-mediated mechanisms to destroy tumor cells. It should be noted that most of the mechanisms of trastuzumab resistance have been identified in preclinical models and have not yet been validated in clinical samples. One important goal for this field is to determine which of the mechanisms outlined are clinically relevant. It is likely that, as with other anticancer agents, clinical resistance will be multifactorial.

Obstacles for trastuzumab binding to HER2

p95HER2. A constitutively active, truncated form of HER2 receptor, p95HER2, has kinase activity but lacks the extracellular domain and the binding site of trastuzumab (Fig. 3A). The expression of full-length (p185) and truncated (p95) HER2 was evaluated on 337 primary breast cancer samples and 81 samples from metastatic lymph nodes using Western blot (62). Truncated p95HER2 was present in 2.1% of the p185HER2-negative tumors, in 19.5% of low, in 31.8% of moderate, and in 60% of tumors that were highly positive for p185HER2 ($P < 0.001$). A higher proportion of node-positive patients than node-negative patients express p95HER2 (44, 62).

Although *in vitro* data suggested trastuzumab could block the cleavage of p185HER2 and consequent production of constitutively active p95HER2 (27), a retrospective study revealed a strong association between the presence of p95HER2 and clinical resistance to trastuzumab treatment (63). In that study, sensitivity to trastuzumab was evaluated according to p95HER2 expression measured by immunofluorescence in 46 patients with metastatic breast cancer. Only 1 out of 9 patients (11.1%) expressing p95HER2 responded to trastuzumab, whereas 19 of the 37 patients (51.4%) with tumors expressing p185HER2 achieved clinical response.

On the other hand, trastuzumab binds to the extracellular domain and forms a trastuzumab-extracellular domain complex that was found to have faster clearance than free trastuzumab in preclinical models and in a population pharmacokinetics study (61). In a phase II trial evaluating trastuzumab single agent in HER2-overexpressing metastatic breast cancer, extracellular domain HER2 plasma concentrations greater than 500 ng/mL were associated with shorter serum half-life and subtherapeutic trough levels of trastuzumab (64). Extracellular domain HER2 level has since been evaluated as potential predictor for treatment response, but a pooled analysis of four clinical trials in which trastuzumab was used in metastatic breast cancer (65) revealed that both baseline extracellular domain levels and extracellular domain drop following therapy had low predictive values for clinical benefit with trastuzumab therapy.

Lapatinib, a small molecule that inhibits both HER2 and EGFR kinases, was tested by Scaltriti and colleagues (63) in p95HER2 preclinical models as a strategy to prevent HER2 signaling despite loss of the trastuzumab binding site. Treatment of p95HER2-positive MCF-7 cells with lapatinib-inhibited p95HER2 phosphorylation, reduced downstream phosphorylation of Akt and MAPK, and inhibited cell growth. It also inhibited growth of MCF-7^{p95HER2} xenograft tumors. By contrast, trastuzumab had no effect on this model. The addition of lapatinib to chemotherapy was then evaluated for treatment of patients with stage IV HER-overexpressing breast cancer that was progressive after chemotherapy and trastuzumab. In a phase III trial (66), the addition of lapatinib to capecitabine produced an improvement in the median time to progression, when compared with the use of chemotherapy alone, resulting in FDA approval of this combination for the treatment of patients with advanced or metastatic breast cancer whose tumors overexpress HER2 and who have received prior therapy including an anthracycline, a taxane, and trastuzumab. Lapatinib has been also tested alone or in combination with trastuzumab for patients with

HER2-overexpressing metastatic breast cancer after progression on trastuzumab, with improved progression-free survival for the combination arm (67). The combination trastuzumab-lapatinib is currently being tested in the neoadjuvant and adjuvant settings (68).

Mucin 4. Epitope masking has also been investigated as a mechanism of resistance to trastuzumab. *Mucin 4 (MUC4)* is large, highly O-glycosylated membrane-associated glycoprotein (69), which may interfere with trastuzumab binding to the HER2 receptor (Fig. 3B). In a preclinical model using the human HER2 positive JIMT-1 cell line, which is primarily resistant to trastuzumab, Nagy and colleagues (70) observed that the presence of MUC4 was associated with trastuzumab epitope masking and decreased antibody-binding capacity. In that model, JIMT-1 resistance to trastuzumab could be reversed using RNA interference to knockdown MUC4.

CD44/hyaluronan polymer complex. CD44 is a transmembrane receptor for hyaluronan. Binding of polymeric hyaluronan activates CD44-mediated signal transduction pathways including RAS and PI3K in ovary cancer cells (71). CD44 and hyaluronan may also hinder the access of trastuzumab to HER2 receptor by masking its cognate epitope, and lead to treatment resistance (Fig. 3B). Palyi-Krekk and colleagues (72) showed that treating JIMT-1 cells with an inhibitor of the hyaluronan synthesis significantly reduces the hyaluronan levels of JIMT-1 cells both *in vivo* and *in vitro*, leading to increased binding of trastuzumab to HER2 and its subsequent antitumoral effects. Ghatak and colleagues (73) showed that the binding of endogenous hyaluronan polymer to CD44 contributes to PI3K/Akt activation, but it was not clear if the described Akt activation was dependent on HER2. Blockage of hyaluronan polymer-CD44 binding by anti-CD44 antibodies or by hyaluronan oligomers caused suppression of the PI3K/Akt pathway with consequent inhibition of anchorage-independent growth of cells in culture and of tumors *in vivo*.

Upregulation of HER2 downstream signaling pathways

PTEN. The loss of function of PTEN caused by mutation of *PTEN* itself, or by transcriptional regulation, has been described in several tumors and in up to 50% of breast cancers (74). PTEN normally inhibits the activation of PI3K. Therefore PTEN loss results in the constitutive upregulation of PI3K/Akt (Fig. 4; ref. 75). Nagata and colleagues (39) showed that decreased levels of the PTEN phosphatase resulted in increased PI3K/Akt phosphorylation and signaling, preventing trastuzumab-mediated growth arrest of HER2-overexpressing breast cancer cells. In PTEN-deficient models however, PI3K inhibitors rescued trastuzumab resistance *in vitro* and *in vivo*. Patients with PTEN-deficient HER2-overexpressing metastatic breast cancer had significantly poorer responses to trastuzumab-based therapy than those with tumors expressing normal *PTEN* (39, 74).

PI3K. PI3K mutations have also been implicated in trastuzumab resistance through PI3K/Akt pathway activation (Fig. 4). The *PIK3R1* gene encodes the PI3K regulatory subunit p85 α . *PIK3R1* mutations affect p85 function, inducing constitutive activation of the PI3K/Akt pathway (76, 77). In addition, *PIK3CA*, the gene that encodes the catalytic subunit of p100 α of PI3K, is also frequently mutated or overexpressed in human cancer (78). Using

a baculovirus production system, Huang and colleagues (79) showed that many of the gain-of-function mutations of human p110 α and p85 occur at residues lying at the interfaces between them or between the kinase domain of p110 α and other domains within the catalytic subunit. Additionally, Berns and colleagues (80) described significantly improved ability to detect patients with low response to trastuzumab in a cohort of 55 patients with

breast cancer when combining the analysis of low *PTEN* expression and the presence of oncogenic *PIK3CA* mutations.

Akt and PDK1 Changes. Increased Akt phosphorylation has been described as a mechanism for Akt activation and implicated with shorter survival in a cohort of 61 patients with *de novo* acute myeloid leukemia (81), but this mechanism was not confirmed in a cohort of 45 patients with HER2-positive metastatic

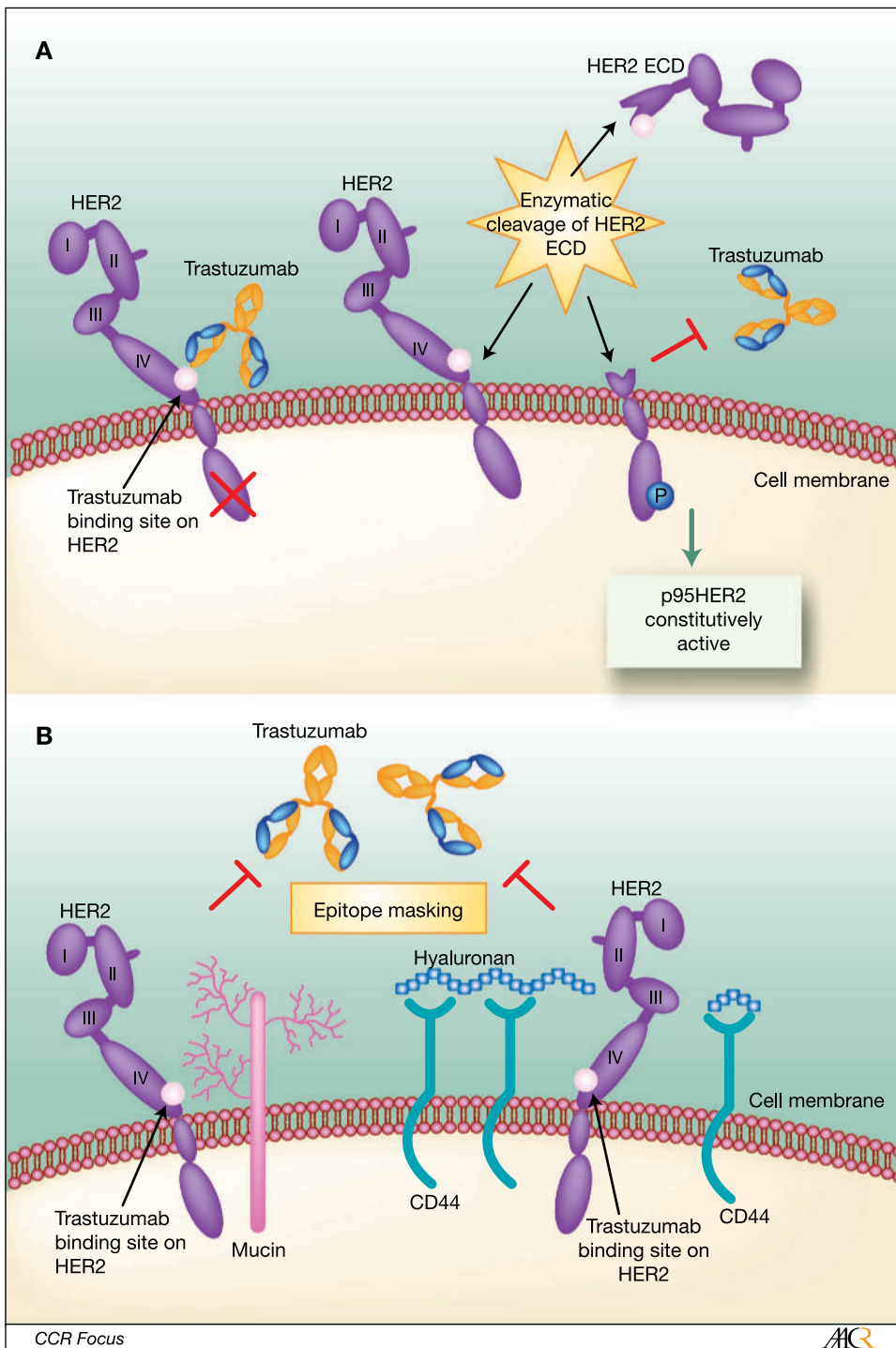


Fig. 3. General mechanisms of resistance to trastuzumab binding to HER2. **A**, a constitutively active truncated form of HER2 receptor that has kinase activity but lacks the extracellular domain and the binding site of trastuzumab is originated from metalloprotease-dependent cleavage of the full-length HER2 receptor (p185; refs. 45, 130). Trastuzumab does not bind to p95HER2 and therefore has no effect against it. The remaining intracellular domain of p95HER2 has operational kinase domains and can be targeted by the TK inhibitor lapatinib (63). **B**, epitope masking by MUC4 or CD44/polymeric hyaluronan complex. MUC4 is a large membrane-associated mucin produced by epithelia as part of the epithelial protective mechanisms. MUC4 has multiple repeat regions containing serine and threonine. Glycosylation of these repeats forces them into a highly extended, rigid conformation and makes them hydrophilic (69). MUC4 is normally present on the apical surfaces of epithelial cells, but overexpressed in several carcinomas. MUC4 has close association with HER2 and may mask trastuzumab cognate epitope, interfering with antibody binding and activity. CD44/hyaluronan polymer complex activates RAS and PI3K pathways, but it is not clear if these effects depend on HER2. Inhibition of hyaluronan synthesis *in vitro* reduces hyaluronan polymer binding to CD44, and increases trastuzumab binding to HER2.

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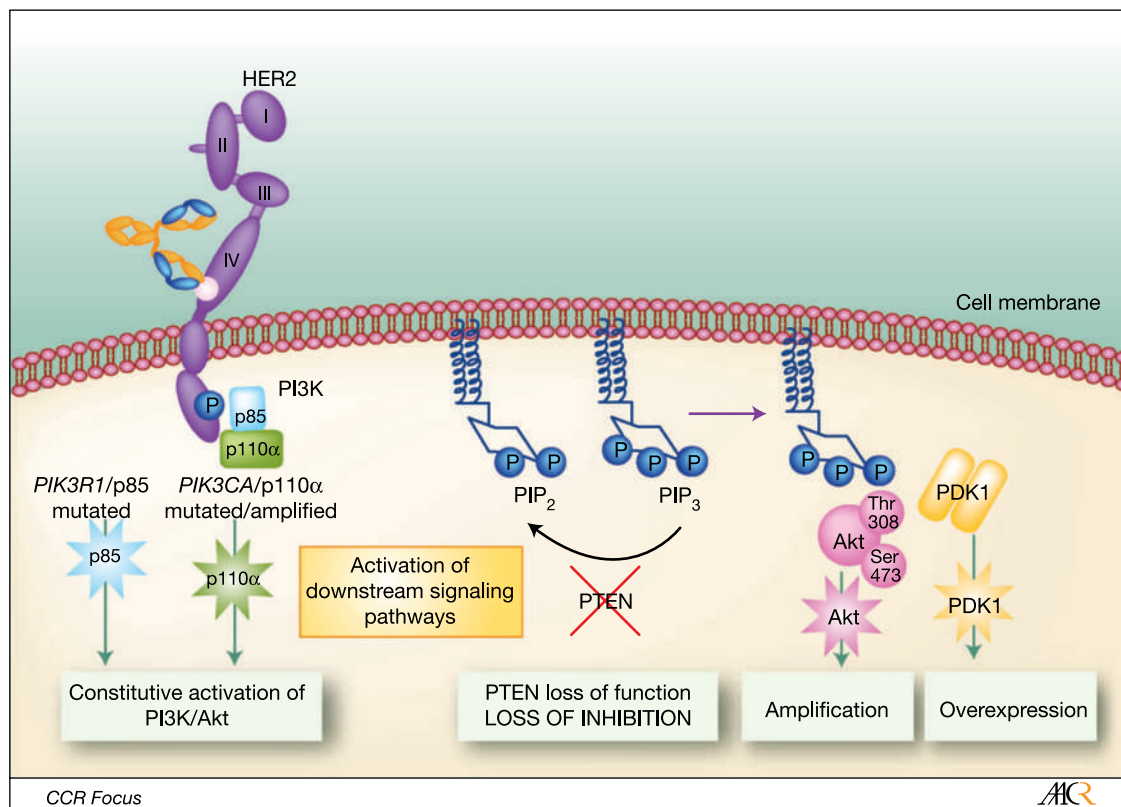


Fig. 4. General mechanisms of resistance to trastuzumab: presence of upregulation of HER2 downstream signaling pathways. PTEN is a tumor suppressor. Trastuzumab binding stabilizes and activates PTEN and consequently down-regulates the PI3K/Akt signaling pathway (39). When PTEN function is lost, PI3K remains constitutively active regardless of binding of trastuzumab to HER2. PTEN loss correlates with clinical unresponsiveness to trastuzumab treatment. Genomic aberrations in the PI3K pathway are a common event in a variety of cancer types (127). Multiple components of this pathway are affected by germline or somatic mutation, amplification, rearrangement, methylation, overexpression, and aberrant splicing, but only in a few instances *PIK3CA* and *PTEN* mutations are seen simultaneously (86). Genomic aberrations in the PI3K pathway produce constitutive activation of the pathway, which will signal downstream to the nucleus regardless of trastuzumab binding to HER2. This is the case with activating mutations of *PIK3R1* and *PIK3CA*, encoding genes for PI3K p85 α and p110 α , respectively. Increased Akt kinase activity and *PDK1* overexpression have also been implicated with trastuzumab resistance.

breast cancer treated with trastuzumab, when pAkt was evaluated by immunohistochemistry (82). Trastuzumab-resistant BT474 cells generated by continuous culture of previously sensitive cells in trastuzumab-containing medium have elevated levels of phosphorylated Akt and Akt kinase activity as compared with BT474 parental cell line (83). These resistant cells also showed increased sensitivity to PI3K inhibitors. An activating mutation of *AKT1* situated in the pleckstrin homology domain (E17K) was identified in breast cancer and occurs early in the development of the disease (84). However, so far this mutation has not been identified in HER2-overexpressing tumors (85). Overexpression of PDK1 was found in approximately 20% of breast cancers (86). Tseng and colleagues (87) evaluated the effects of concurrent use of trastuzumab and a PDK-1 inhibitor in a preclinical model using trastuzumab-resistant insulin-like growth factor-1 receptor (IGF-IR)-overexpressing SKBR3 cells, generating data indicating that PDK-1 inhibition reversed the trastuzumab-resistant phenotype.

Signaling through an alternate receptor pathway

Signaling through multiple alternate pathways has been linked to trastuzumab resistance in various preclinical models.

Clinical information confirming the importance of most of the identified pathways is needed.

Insulin-like growth factor-1 receptor. Another transmembrane TK receptor that stimulates cell proliferation, IGF-1R, was found to interact with HER2 in trastuzumab-resistant cell lines, inducing its phosphorylation (Fig. 5). In these preclinical models, when anti-IGF-IR drugs were used, they restored sensitivity to trastuzumab-resistant cells (88). Lu and colleagues (89, 90) showed that the trastuzumab-induced growth inhibition in HER2-overexpressing cells can be compensated for by increased IGF-IR signaling, resulting in resistance to trastuzumab. IGF-IR-mediated resistance to trastuzumab treatment seems to involve the PI3K pathway, leading to enhanced degradation of p27 (89, 91).

Harris and colleagues (92) carried on a clinical trial evaluating clinical benefits and potential predictors of response to neoadjuvant trastuzumab plus vinorelbine in patients with operable HER2-overexpressing breast cancer. In this trial single and multigene biomarkers were evaluated. Notably, the presence of IGF-IR membrane expression was associated with a lower response rates (50% versus 97%; $P = 0.001$). These results suggest that HER2-overexpressing tumors with co-expression of

IGF-IR are more likely to be resistant to this trastuzumab-containing regimen.

p27. As a Cdk inhibitor involved in regulating cell proliferation, p27 is a distal downstream regulator of multiple converging growth factor receptor pathways including EGFR, HER2, and IGF-IR. Nahta and colleagues (93) showed that HER2-overexpressing trastuzumab-resistant cells have low p27 levels, low p27/Cdk2 complexes, and, thus, increased Cdk2 activity and proliferation rate. When p27 expression was increased by transfection or pharmacological induction with a proteasome inhibitor, trastuzumab sensitivity was restored. Consistent with data that implicates Akt in p27 regulation, Yakes and colleagues (26) showed that Akt inhibition is at least partially responsible for the changes in cell cycle- and apoptosis-regulatory molecules after HER2 blockade with trastuzumab.

EGFR and HER3. EGFR homodimers and EGFR/HER3 heterodimers, lacking HER2, could potentially bypass trastuzumab blockade. However, these dimers are less likely to be the driving force in HER2-overexpressing breast cancer, because HER2 is the preferred heterodimer partner of this family of receptors (94). In a recent study examining trastuzumab-resistant breast cancer cells to gain insights into trastuzumab resistance mechanisms, both EGFR and HER3 expression were increased after long-term trastuzumab exposure in culture (95). Interestingly, chronic trastuzumab exposure of trastuzumab-resistant cell lines induced sensitivity to anti-EGFR agents. Unfortunately, a phase I-II clinical trial designed to evaluate safety and efficacy of the EGFR TK inhibitor gefitinib with weekly trastuzumab suggested that the combination was unlikely to result in clinical

benefit when compared with trastuzumab alone (96). On the other hand, as previously mentioned, lapatinib, an EGFR/HER2 TK inhibitor, has clinical activity in the treatment of patients who progressed on trastuzumab (66), but this effect may result from its ability to inhibit HER2 TK.

HER3 is the favored receptor for dimerization with HER2 (94), and growing evidence supports HER3 as being a required partner for HER2 in HER2-overexpressing breast cancer (40, 97). Investigating the oligomerization properties of the ErbB receptors, Wehrman and colleagues (42) confirmed that interactions between EGFR/HER2 and HER2/HER3 are detected in the presence of ligand. In addition, they found that trastuzumab is ineffective in blocking HER2/HER3 dimerization.

Pertuzumab, an anti-HER2 humanized mAb that binds to an epitope located in domain II of HER2, sterically blocks a binding pocket necessary for receptor dimerization and signaling. It therefore prevents HER2 dimerization, including HER2/HER3 heterodimerization. Pertuzumab is active against HER2-overexpressing cell lines and xenografts, and preclinical data suggest potential synergism with trastuzumab (98). Preliminary results from a phase II trial of pertuzumab in combination with trastuzumab in patients with HER2-overexpressing metastatic breast cancer with disease progression on trastuzumab have suggested good tolerance and clinical activity of the combination (99). Clinical trials testing pertuzumab in combination with trastuzumab in different settings, as well as pertuzumab with chemotherapy are ongoing (68).

c-Met. Frequently co-expressed with HER2 in cell lines, the c-Met receptor may contribute to trastuzumab resistance

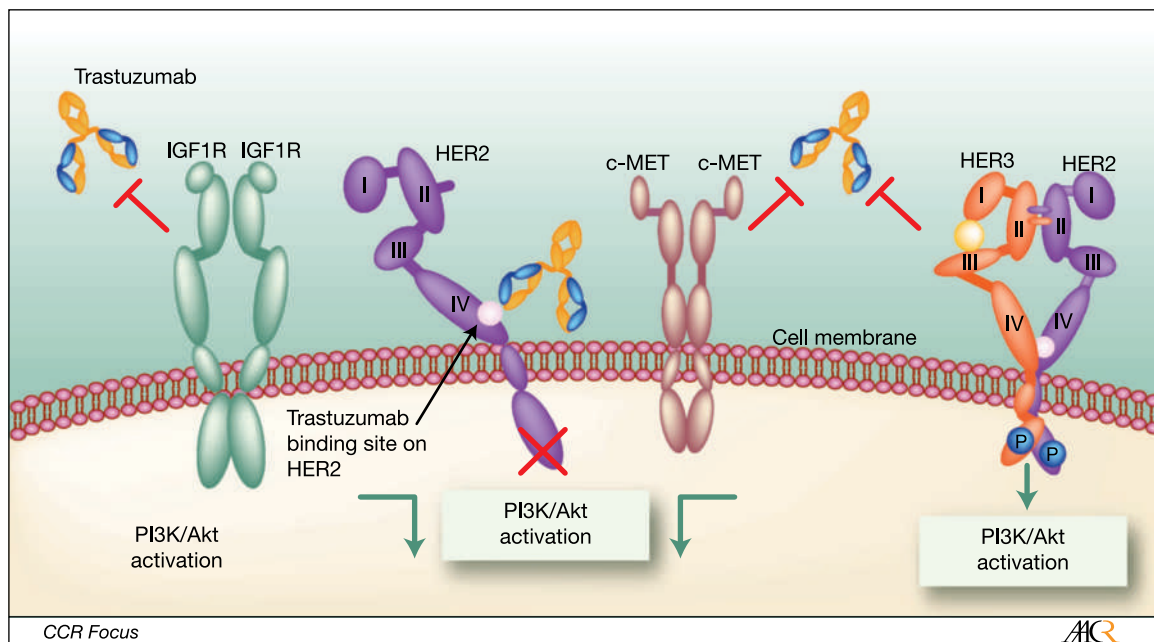


Fig. 5. General mechanisms of resistance to trastuzumab: presence of signaling through an alternate receptor and/or pathway. Signaling may continue regardless of trastuzumab binding to HER2 when other receptors remain active on the tumor cell. The activity of certain receptors may also increase as a result of trastuzumab blockage of HER2, as a cell survival mechanism (see text for details). Trastuzumab-induced growth inhibition in HER2-overexpressing cells can be compensated for by increased IGF-IR signaling, resulting in resistance to trastuzumab. In preclinical models in which HER2-overexpressing tumor cells are cultured in the presence of ligand, resembling what is likely to happen *in vivo*, trastuzumab does not interfere with HER2/HER3 heterodimerization and therefore does not block signaling from these heterodimers (42). c-Met is frequently co-expressed with HER2 in cell lines and contributes to trastuzumab resistance through sustained Akt activation.

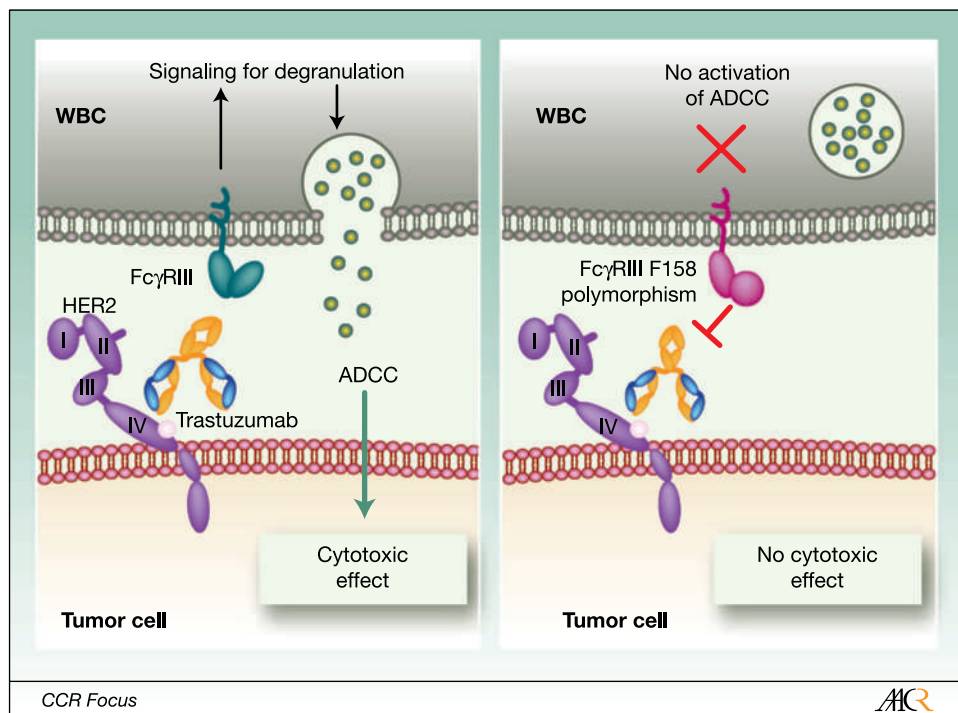


Fig. 6. General mechanisms of resistance to trastuzumab: failure to trigger immune-mediated mechanisms to destroy tumor cells. ADCC is a process in which the Fab region of an antibody binds to its cognate antigen present on a target cell (for instance cancer cell), whereas its Fc region engages with the Fc receptor present on an effector cell from immune system. This process triggers degranulation of cytotoxic granules from effector cell toward the target cell and culminates with target cell apoptosis (131). In humans, the Fc receptor family comprises FcγRI (CD64); FcγRII (CD32), with three isoforms FcγRIIIa, FcγRIIIb (inhibitory), and FcγRIIIc; and FcγRIII (CD16), including two isoforms FcγRIIIa and FcγRIIIb (132). There is a correlation between the clinical efficacy of therapeutic antibodies in humans and their allotype of high-affinity (V158) or low-affinity (F158) polymorphic forms of FcγRIIIa. Epitope masking previously discussed in the obstacles for trastuzumab binding to HER2 section would also play a role by preventing antibody-based cell destruction.

through sustained Akt activation (Fig. 5). HER2-overexpressing breast cancer cells respond to trastuzumab with a rapid upregulation of c-Met receptor expression, and c-Met activation protects cells against trastuzumab (100). Loss of c-Met function produced through RNA interference improves the response of these cell lines to trastuzumab.

CXCR4, $\alpha6\beta1$, and $\alpha6\beta4$ integrins. Acquired resistance to trastuzumab was associated with CXCR4 upregulation and nuclear redistribution. Inhibition of CXCR4 reversed acquired trastuzumab resistance *in vitro* (101). *In vitro* experiments suggest both $\alpha6\beta1$ and $\alpha6\beta4$ integrins may be involved in *de novo* and/or acquired resistance to targeted therapy against HER2 (102).

Failure to trigger immune-mediated mechanisms to destroy tumor cells

Fc, Fc receptor, and ADCC. A genomic polymorphism producing the phenotype expression of valine (V) or phenylalanine (F) at amino acid 158 on the FcγRIIIa significantly influences the affinity of IgG1 to the Fcγ receptor (Fig. 6; ref. 103). Immune effector cells carrying the FcγRIIIa V/V alleles mediate ADCC of trastuzumab and anti-HER2/*neu* IgG1 variants better than cells bearing the F allele (60). In the clinic, the FcγRIII 158V/F polymorphism interfere with the ability to generate ADCC responses *in vitro* during trastuzumab treatment and significantly impair clinical response

rates and progression-free survival of patients treated with trastuzumab in the metastatic setting (30). In an attempt to improve FcγRIIR binding, Lazar and colleagues (104) used a combination of computational structure-based protein design methods coupled with high-throughput protein screening to optimize the FcγR binding capacity of therapeutic antibodies. The described engineered Fc variants with improved FcγR affinity and specificity had enhanced cytotoxicity *in vitro*, with improved affinity and ADCC for both the V158 and F158 forms of FcγRIIIa. The authors conclude that this progress has promising implications for therapeutic mAb design, with potential future expanding of the population of patients responsive to anticancer antibody-based treatment (104).

Recent additional evidence for the importance of immune-mediated mechanisms in antibody-based therapy of HER2-overexpressing tumors came from a lapatinib preclinical study by Scaltriti and colleagues (105). The authors treated SKBR3 and MCF7 cells with lapatinib alone or in combination with trastuzumab, producing inhibition of HER2 phosphorylation and preventing receptor ubiquitination, which resulted in a marked accumulation of inactive HER2 receptors at the cell surface. Trastuzumab alone caused degradation of the receptor. Lapatinib-induced accumulation of HER2 in tumors was also observed in BT474 xenografts. In mice trastuzumab-lapatinib combination produced complete tumor remissions in all cases

after 10 days of treatment. Accumulation of HER2 at the cell surface by lapatinib was shown to enhance immune-mediated trastuzumab-dependent cytotoxicity.

Further evidence on the importance of ADCC was provided by Barok and colleagues (29), who showed that trastuzumab can trigger ADCC and destroy trastuzumab-resistant HER2 positive cell lines and xenografts. Taken together, these observations point to ADCC as a major player in trastuzumab antitumor effect, at least when the antibody binds to HER2. Thus far the ADCC-triggered pathways on target cells have not received much attention, but it would be very interesting to know how ADCC affects cells that are prone to enhance PI3K/Akt and RAS/Raf/MAPK pathways to an extreme, in order to survive.

Trastuzumab clearance mechanisms are not well described. Multiple proteases involved with infection, inflammation, and tumor environments have been shown to be able to cleave IgG molecules on site, generating monovalent or bivalent antibody fragments, Fab' or F(ab')₂, which lack Fc-mediated effector function (106). These antibody fragments can still bind to their cognate antigen, but lack binding sites for complement and for Fc receptors, and can no longer trigger Fc-mediated functions such as ADCC. Although cleavage of HER2 is well documented, it is uncertain if trastuzumab undergoes cleavage on tumor site as well.

Discussion

Trastuzumab is an essential and effective targeted anticancer agent. It has been moved into first-line treatment of HER2-overexpressing breast cancer both in the adjuvant and metastatic settings. As an antibody, trastuzumab has several properties,

either related to its Fab and/or Fc function. Unfortunately, a significant number of patients develop progressive disease after initial treatment with trastuzumab-containing regimens, requiring additional therapy.

At the diagnosis of progressive disease after trastuzumab treatment, reasonable options for patients are: (1) participation on clinical trials containing various combinations of trastuzumab and/or lapatinib with PI3K/Akt pathway inhibitors, other TK inhibitors, IGF-1 inhibitors, cell cycle regulators, mammalian target of rapamycin (mTOR) inhibitors, anti-angiogenic therapy or chemotherapy; (2) participation on clinical trials evaluating vaccines, other anti-HER2 antibodies such as pertuzumab, or anti-HER2 immunotoxins; (3) continuing anti-HER2 therapy including trastuzumab beyond progression with a different chemotherapy regimen (107); or (4) switching to a lapatinib containing regimen (66, 68). The observation of clinical benefit of the addition of trastuzumab to second-line chemotherapy suggests HER2 blockade is still important for some patients. This can be currently achieved in clinical practice with trastuzumab or lapatinib.

Based on emerging knowledge of the HER2-signaling pathways and of signaling through alternate pathways, several drugs directed to HER2-overexpressing breast cancer are in different phases of clinical development, whereas others are still struggling to leave the bench (Table 1). In the case of anti-HER2 therapy, except for the presence of the HER2 itself, there are no validated biomarkers reliably predicting benefit from trastuzumab that can be used for either clinical trial development or individual therapeutic decisions, because it is unclear which of the mechanisms of action or resistance to trastuzumab are more important in each patient subset. In addition, resistant cells present deletions, insertions, and missense point mutations that influence the function of receptors, adaptor proteins, and

Table 1. New anti-HER2 agents

General strategy	Identification	Target	Reference
Inhibiting downstream signaling pathways of HER2	Perifosine	PI3-kinase/Akt	108
	Everolimus	mTor	109
	GDC-0941	PI3K	43
HER family TK inhibitors	Lapatinib	Dual TK inhibitor of EGFR and HER2 approved for clinical use	110, 111
	Neratinib (HKI-272)	TK inhibitor: HER1/HER2	110
	BIBW2992	Irreversible dual HER1/HER2 inhibitor	112
Inhibiting the activity of other growth factor receptors	Pertuzumab	HER2/HER3	41
	rhIGFBP-3	IGF-1R	113
	IMC-A12	IGF-1R	114
	BMS-554417	IGF-1R	115
	SU11274	MET	100
Decreasing the expression of protein processing of HER2 and other signaling components	Tanespimycin (17-AAG)	HSP90 inhibitor	116
	LAQ824	Histone deacetylase (HDAC) inhibitors	117
	Bortezomib	Proteasome inhibitor	118
Increasing toxicity of trastuzumab to HER2 positive cells	Trastuzumab-DM1 immunotoxin	HER2	119
	HER2 vaccines	Anti-HER2 vaccine	120
Increasing immunity against HER2 positive cells	Ertumaxomab	Trifunctional bispecific antibody targeting HER2 and CD3	121
	Genetically engineered Fc of mAb	Enhanced FcγRIII receptor binding on effector cells	104

second messengers, allowing for tumor cell survival in the presence of trastuzumab. Tumor cells seem to be highly wired and activate or up-regulate alternative survival pathways upon treatment with a single form of targeted therapy. For this reason the inhibition of one deregulated protein is unlikely to be enough to solve the trastuzumab resistance problem. To add to this complex scenario, cellular pathways from HER2 activation are redundant, and activation of escape mechanisms by resistant cells may also involve constantly switching host-tumor environmental characteristics.

The challenge is to prioritize strategies for faster development. Several compounds have been tested in preclinical models, but only a few are currently moving on into the clinical development platforms. It is critical that this field begin to move from the *in vitro* descriptions of mechanism to clinical validation. The study of the incidence of abnormalities on the analyzed pathways could help with selecting priorities for development. Brugge and colleagues (86) produced a list of aberrations in the PI3K pathway or in its interacting pathways that have been described in cancer, with estimations of number of cases involved with each aberration per year in the United States.

Another strategy would include advancing clinical assay development, permitting accurate diagnosis of the implicated resistance mechanism in each subset of patients. The development of such assays and of the corresponding targeted therapy should preferably occur simultaneously. Once the main trastuzumab resistance mechanism is identified for a subgroup of patients, this information can be used for the design of subsequent clinical trials. On the other hand, the clinical trials would aid on clinical assay study and validation.

In this scenario, for patients who present with trastuzumab resistance owing to interference with trastuzumab binding, a strategy to overcome resistance may involve the use of treatments that do not depend on molecular binding to the HER2 extracellular domain, including the use of TK inhibitors such as lapatinib (63), or of inhibitors of the downstream signaling pathways of HER2. If p95HER2 is thought to be the driving force for resistance to trastuzumab in a subgroup of patients, pertuzumab, trastuzumab-DM1, trastuzumab-mediated ADCC, and HER2-extracellular domain-based vaccines may not be an option. Other patients may present with tumors containing PTEN loss, *PIK3R1/p85 α* , *PIK3CA/p110 α* mutations or amplification, as well as activation of alternate pathways. If combined, these alterations may complicate the targeting of intracellular molecules, as they would require the simultaneous use of different inhibitors. In this case, reasonable options would be to increase tumor cell destruction through extracellular mechanisms by increasing or enabling ADCC, or by using immunotoxins such as the antibody-drug conjugate trastuzumab-DM1, or the bispecific anti-HER2/CD3 antibody ertumaxomab.

Thus, understanding trastuzumab resistance mechanisms is critical for the educated development of strategies to overcome it, as well as for the development of tools that would allow definitive and efficient patient selection for therapies that could mitigate that resistance.

Disclosure of Potential Conflicts of Interest

The authors have no potential conflicts of interest to disclose.

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