Review
HER2 therapy
Molecular mechanisms of trastuzumab resistance

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Abstract

Trastuzumab is a monoclonal antibody targeted against the HER2 tyrosine kinase receptor. The majority of patients with metastatic breast cancer who initially respond to trastuzumab develop resistance within one year of treatment initiation, and in the adjuvant setting 15% of patients still relapse despite trastuzumab-based therapy. In this review, we discuss potential mechanisms of antitumor activity by trastuzumab, and how these mechanisms become altered to promote therapeutic resistance. We also discuss novel therapies that may improve the efficacy of trastuzumab, and that offer hope that the survival of breast cancer patients with HER2-overexpressing tumors can be vastly improved.

Introduction

Approximately 20% to 25% of invasive breast cancers exhibit overexpression of the human epidermal growth factor receptor (HER)2 tyrosine kinase receptor [1,2]. As elevated HER2 levels are associated with reduced disease-free and overall survival in metastatic breast cancer (MBC) [1,3], therapeutic strategies are being developed to target this oncoprotein. Trastuzumab (Herceptin®; Genentech, South San Francisco, CA, USA), a recombinant humanized monoclonal antibody (rhumAb 4D5) directed against an extracellular region of HER2 [4], was the first HER2-targeted therapy approved by the United States Food and Drug Administration (FDA) for the treatment of HER2-overexpressing MBC. In addition, trastuzumab with adjuvant chemotherapy (either in sequence or in combination) significantly improved disease-free and overall survival rates in patients with early stage HER2-overexpressing breast cancer [5-7].

Trastuzumab: mechanisms of antitumor effects

The mechanisms by which trastuzumab induces regression of HER2-overexpressing tumors are still being elucidated, but several molecular and cellular effects have been reported in the literature [8].

Trastuzumab reduces signaling mediated by HER2 through the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) cascades. Reduced downstream signaling through these pathways induces the cyclin-dependent kinase inhibitor p27kip1, which promotes cell-cycle arrest and apoptosis [9,10].

Trastuzumab rapidly dissociates the non-receptor tyrosine kinase Src from HER2, reducing Src activity such that the phosphatase and tensin homolog deleted on chromosome ten (PTEN) is dephosphorylated and translocated to the plasma membrane where it is active [11]. The PI3K downstream effectors Akt and mammalian target of rapamycin (mTOR) are then inhibited.

The efficacy of trastuzumab may also depend upon its ability to induce an immune response. HER2-targeted antibodies, including trastuzumab, were shown to promote apoptosis in multiple breast cancer cell lines via antibody-dependent cellular cytotoxicity (ADCC) [12-15]. Importantly, mice that were null for the Fc gamma receptor expressed on natural killer cells, which are important for ADCC, lost much of the antitumor effect of trastuzumab, with only 29% tumor growth

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ADCC = antibody-dependent cellular cytotoxicity; ECD = extracellular domain; EGF = epidermal growth factor; EGFR = epidermal growth factor receptor; ER = estrogen receptor; FDA = Food and Drug Administration; HER = human epidermal growth factor receptor; IGF = insulin-like growth factor; IGF-IR = insulin-like growth factor-I receptor; MAPK = mitogen-activated protein kinase; MBC = metastatic breast cancer; mTOR = mammalian target of rapamycin; PI3K = phosphatidylinositol 3-kinase; PTEN = phosphatase and tensin homolog deleted on chromosome ten.

inhibition observed versus 96% in control mice expressing the Fc gamma receptor and with intact natural killer cell function [13]. Thus, an active immune response to trastuzumab may be partially responsible for cytotoxic activity. Furthermore, a higher *in situ* infiltration of leukocytes and ADCC activity were observed in patients achieving complete or partial remission after receiving preoperative trastuzumab relative to those who did not respond to this regimen [14]. Since patients with advanced MBC are immunosuppressed, it is difficult to appreciate the magnitude of the contribution of ADCC to trastuzumab-mediated tumor inhibition. More in-depth *in vivo* studies are required to grasp exactly how important the contribution of ADCC is to mediating the response to trastuzumab and whether other targeted antibodies used against solid tumors also rely upon immune modulation to achieve response.

Trastuzumab has also been shown to inhibit angiogenesis, resulting in decreased microvessel density *in vivo* [16-18] and reduced endothelial cell migration *in vitro* [17]. Expression of pro-angiogenic factors was reduced, while expression of anti-angiogenic factors was increased in trastuzumab-treated tumors relative to control-treated tumors *in vivo* [16-18]. Combining trastuzumab with the chemotherapeutic agent paclitaxel actually inhibited angiogenesis more potently than did trastuzumab alone [17], perhaps due to trastuzumab-mediated normalization of the tumor vasculature allowing for better drug delivery [16].

Trastuzumab: clinical efficacy and resistance

Trastuzumab is active as a single agent and in combination with chemotherapy in HER2-overexpressing MBC, leading to FDA approval of trastuzumab in 1998 for treatment in this setting. The objective response rates to trastuzumab monotherapy were low, ranging from 12% to 34% depending on prior therapy for metastatic disease, for a median duration of 9 months. Hence, the majority of HER2-overexpressing tumors demonstrated primary (*de novo* or intrinsic) resistance to single-agent trastuzumab. In fact, the rate of primary resistance to single-agent trastuzumab for HER2-over-expressing MBC is 66% to 88% [19-21]. Further phase III trials revealed that combining trastuzumab with paclitaxel [22,23] or docetaxel [24] could increase response rates, time to disease progression, and overall survival compared with trastuzumab monotherapy. In patients whose tumors had amplified *her2* and had not received prior chemotherapy for MBC, the median time to progression in response to single-agent trastuzumab treatment was 4.9 months [22]; in patients who received trastuzumab and chemotherapy, the median time to progression was 7.4 months [23]. Thus, the majority of patients who achieve an initial response to trastuzumab-based regimens develop resistance within one year. In the adjuvant setting, administration of trastuzumab in combination with or following chemotherapy improves the disease-free and overall survival rates in patients with early stage breast cancer [5-7]. However, approximately 15% of these women

still develop metastatic disease despite trastuzumab-based adjuvant chemotherapy. Elucidating the molecular mechanisms underlying primary or acquired (treatment-induced) trastuzumab resistance is critical to improving the survival of MBC patients whose tumors overexpress HER2 (Table 1) [25].

Trastuzumab: mechanisms of resistance
Steric hindrance of receptor-antibody interaction: overexpression of MUC4

A potential mechanism by which resistance to targeted antibodies may develop is via disruption of the interaction between the therapeutic agent and the target protein. Resistance to trastuzumab was associated with increased expression of the membrane-associated glycoprotein MUC4 [26]. MUC4 was shown to bind and sterically hinder HER2 from binding to trastuzumab [26,27]. MUC4 has been suggested to contribute to cancer because of its ability to inhibit immune recognition of cancer cells, promote tumor progression and metastasis, suppress apoptosis, and activate HER2 [28]. MUC4 interacts directly with HER2, an event that is dependent upon an epidermal growth factor (EGF)-like domain on the ASGP-2 subunit of MUC4 [26]. Through this interaction, it is proposed that MUC4 serves as a ligand for HER2, resulting in increased phosphorylation of HER2 on the residue Tyr1248 [26], which is a major phosphorylation site contributing to the transforming ability of the HER2 oncoprotein [29]. MUC4 does not affect total HER2 receptor expression levels [26,28]. The JIMT-1 trastuzumab-resistant cell line described by Nagy and colleagues [27] was established from a breast cancer patient showing *her2* gene amplification and primary resistance to trastuzumab [30]. Using this model, the authors demonstrated that the level of MUC4 protein was inversely correlated with the trastuzumab binding capacity, and showed that knockdown of MUC4 increased the sensitivity of JIMT-1 cells to trastuzumab [27]. Thus, the authors proposed that elevated MUC4 expression masks the trastuzumab binding epitopes of HER2, resulting in steric hindrance of the interaction between this antibody and its therapeutic target, resulting in drug resistance. Interestingly, the authors also reported that HER2 was unable to interact with other proteins, such as EGFR or HER3, because of epitope masking by MUC4.

Insulin-like growth factor-I receptor signaling

Trastuzumab resistance has been associated with increased signaling from the insulin-like growth factor-I receptor (IGF-IR). Increased expression of IGF-IR was shown to reduce trastuzumab-mediated growth arrest of HER2-overexpressing breast cancer cells [31]. Expression of IGF-binding protein 3, which blocks IGF-I-mediated activation of IGF-IR, restored trastuzumab sensitivity. We recently demonstrated that crosstalk occurs between IGF-IR and HER2, and showed that IGF-IR physically interacts with and phosphorylates HER2 in trastuzumab-resistant cells, but not in trastuzumab-sensitive

Table 1

Proposed mechanisms of trastuzumab resistance

Mechanism	Example	References
Therapeutic agent cannot recognize molecular target: disrupted interaction between HER2 and trastuzumab	Overexpression of MUC4 sterically hinders antibody from binding HER2 surface receptor and may mediate cross-talk to activate HER2. Knockdown of MUC4 restored trastuzumab sensitivity of breast cancer cells *in vitro*	[26,27]
Compensatory signaling: increased signaling from HER family members	Growth factor ligands of EGFR, HER3, or HER4 (EGF, betacellulin, heregulin) reduced growth inhibitory effect of trastuzumab by 57, 84, and 90 percent, respectively. Trastuzumab binds domain IV of HER2 and domain II is involved in dimerization with ligand-activated family members; trastuzumab did not block heregulin-activated HER3/HER2 interaction in SKBR3 cells	[53,72]
Compensatory signaling: increased signaling from other receptor types	Overexpression of IGF-IR reduced trastuzumab-mediated growth arrest. Inhibition of IGF-I signaling by IGFBP3 increased sensitivity. IGF-IR interacts with and cross-talks to HER2 in trastuzumab-resistant cells but not in sensitive cells. Inhibition of IGF-IR increased trastuzumab sensitivity	[31-33]
Altered downstream signaling	PTEN deficiency correlated with resistance in clinical samples	[11]
	Increased Akt activity	[34,37]
	P27kip1 downregulation	[35,36]
Competition for binding therapeutic agent	Increased circulating HER2 ECD	[40]

ECD, extracellular domain; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HER, human epidermal growth factor receptor; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-I binding protein; IGF-IR, insulin-like growth factor-I receptor; PTEN, phosphatase and tensin homolog deleted on chromosome ten.

parental cells [32]. Our results showed that resistant cells exhibited more rapid IGF-I stimulation of downstream PI3K/Akt and MAPK pathways relative to parental cells. Inhibition of IGF-IR signaling, either by antibody blockade or IGF-IR tyrosine kinase inhibition, restored trastuzumab sensitivity in our *in vitro* resistant model, demonstrating the potential importance of this pathway as a therapeutic target in trastuzumab-resistant breast cancer. Similar to Lu and colleagues [33], we observed downregulation of p27[kip1] upon IGF-I stimulation in both parental and resistant cells [32]. Importantly, antisense oligonucleotides [34] and small interfering RNA [35] that reduced p27[kip1] expression levels also blocked trastuzumab-mediated growth arrest in HER2-overexpressing SKBR3 breast cancer cells. Transfection of p27[kip1] or pharmacological induction of p27[kip1] by the proteasome inhibitor MG132 restored trastuzumab sensitivity in our resistant model [36]. These results suggest that p27[kip1] is a critical mediator of trastuzumab response, and that its downregulation may occur subsequent to increased signaling from growth factor receptors such as IGF-IR, promoting resistance to trastuzumab.

PTEN and PI3K signaling

Growth factor receptor tyrosine kinases, such as HER2 and IGF-IR, activate the PI3K signaling pathway. Constitutive PI3K/Akt activity was previously shown to inhibit cell-cycle arrest and apoptosis mediated by trastuzumab [34]. Furthermore, trastuzumab-resistant cells derived from the BT474 HER2-overexpressing breast cancer line demonstrated elevated levels of phosphorylated Akt and Akt kinase activity compared with parental cells [37]. These resistant cells also showed increased sensitivity to LY294002, a small molecule inhibitor of PI3K. Nagata and colleagues [11] provided compelling evidence supporting a role for the PI3K/Akt pathway in trastuzumab resistance. They demonstrated that decreased levels of the PTEN phosphatase resulted in increased PI3K/Akt phosphorylation and signaling and blocked trastuzumab-mediated growth arrest of HER2-overexpressing breast cancer cells. Importantly, they showed that patients with PTEN-deficient HER2-overexpressing breast tumors have a much poorer response to trastuzumab-based therapy. Furthermore, they showed that, in PTEN-deficient cells, PI3K inhibitors rescued trastuzumab

resistance *in vitro* and *in vivo*. These results suggest that PTEN loss may serve as a predictor of trastuzumab resistance, and that PI3K inhibitors should be explored as potential therapies in patients with trastuzumab-resistant tumors expressing low levels of PTEN protein.

Serum HER2 extracellular domain

The full-length 185 kDa HER2 protein has been reported to be cleaved by matrix metalloproteases into a 110 kDa extracellular domain (ECD), which is released into cell culture media [38-40] or circulating in serum *in vivo* [41-44], and a 95 kDa amino-terminally truncated membrane-associated fragment with increased kinase activity [45]. Elevated serum levels of HER2 ECD correlate with poor prognosis in patients with advanced breast cancer [41-44,46]. Of potential importance, trastuzumab blocked HER2 ECD proteolytic cleavage *in vitro* [47], and patients with elevated pre-treatment ECD levels had higher response rates to trastuzumab [48,49]. HER2 overexpression in breast cancers correlated with elevated pre-treatment levels of circulating HER2 ECD in patients treated with trastuzumab and paclitaxel, and among these patients, responses correlated with a decline in ECD levels over 12 weeks of therapy versus lower responses in those whose ECD levels remained high post-treatment [50].

Zabrecky and colleagues [40] first described the presence of cleaved ECD in the culture medium of HER2-overexpressing SKBR3 breast cancer cells. The authors showed that HER2-targeted monoclonal antibodies bound to circulating ECD, competing away binding to membrane-bound HER2. Hence, signaling from the receptor form of HER2 continued in the presence of HER2 antibodies, indicating that HER2 ECD promoted resistance to HER2-targeted antibody therapy. However, the predictive role of elevated baseline ECD prior to treatment is not well defined. In one study, elevated HER2 ECD levels predicted favorably for response to trastuzumab and docetaxel [24], but other studies showed limited predictive value in this setting. Interestingly, declining levels of circulating HER2 ECD correlate with improved disease-free survival in several studies [24,49]. A meta-analysis of 8 clinical trials revealed that patients whose HER2 ECD levels declined by at least 20% in the first few weeks after initiation of trastuzumab-based therapy had improved disease-free and overall survival compared with patients whose HER2 ECD levels did not drop [51]. Hence, circulating ECD of HER2 may be a serum marker useful for predicting response to trastuzumab. In contrast to these studies, a recent study by Anido and colleagues [52] suggests that truncated forms of HER2 are actually the result of alternative initiation of translation from different methionines within the *her2* sequence, which are referred to as C-terminal fragments of HER2. The authors present compelling *in vivo* data showing that trastuzumab does not inhibit growth of mammary xenografts of the T47D breast cancer cell line stably transfected with the truncated form of HER2, but does inhibit growth of T47D HER2 stable transfectant xenografts. Hence,

this study suggests that the presence of truncated forms of HER2 may promote resistance to trastuzumab.

Novel therapeutic strategies

Trastuzumab resistance is a major clinical problem that requires concentrated effort to resolve. A clear understanding of HER2 and trastuzumab activity at the molecular and biological levels is needed to fully improve survival of patients whose breast cancers overexpress HER2. As these molecular mechanisms begin to be elucidated, more targeted therapies can be developed to improve response rates in the HER2-overexpressing population and in trastuzumab-refractory patients.

Pertuzumab

The recombinant humanized HER2 monoclonal antibody pertuzumab (Omnitarg™, 2C4, Genentech) represents a new class of drugs called dimerization inhibitors; these have the potential to block signaling by other HER family receptors, as well as inhibiting signaling in cells that express normal levels of HER2. Pertuzumab sterically blocks dimerization of HER2 with EGFR and HER3, inhibiting signaling from HER2/HER3 and HER2/EGFR heterodimers [53]. Interestingly, we also observed that pertuzumab disrupted interaction between HER2 and IGF-IR in trastuzumab-resistant cells [32]. Trastuzumab and pertuzumab bind to different epitopes in the extracellular domain of HER2, with trastuzumab binding domain IV of the extracellular domain [54] and pertuzumab binding near the junction of domains I, II, and III of the HER2 extracellular domain [55]. Thus, pertuzumab could theoretically be effective in trastuzumab-resistant tumors. However, while combining trastuzumab with pertuzumab produced synergistic apoptosis in HER2-overexpressing trastuzumab-naïve breast cancer cells [56], this agent failed to demonstrate statistically significant differences on the viability of trastuzumab-resistant breast cancer cells [30,32]. The mechanisms by which trastuzumab-resistant cells develop cross-resistance to alternative HER2-targeted antibodies are unclear, but may reflect aberrations in downstream signaling pathways resulting in resistance to a variety of HER2-targeted agents. Clearly, additional preclinical studies are required to determine the potential efficacy of novel HER2-targeted antibodies in trastuzumab-resistant breast cancers.

Lapatinib

Lapatinib (Tykerb™, GSK572016, formerly GW572016; GlaxoSmithKline, Research Triangle Park, NC, USA) is a dual tyrosine kinase inhibitor targeted against both EGFR and HER2. In comparison to other tyrosine kinase inhibitors in clinical trials (for example, gefitinib, erlotinib), interaction of lapatinib with EGFR and HER2 is reversible, similar to other agents, but dissociation is much slower, allowing for prolonged downregulation of receptor tyrosine phosphorylation in tumor cells. Differences in enzyme-inhibitor structures could account for differences in dissociation off-rate, as EGFR is in a closed conformation

when lapatinib binds versus a more open conformation when gefitinib binds [57]. However, effects on HER2 appear to be more critical to efficacy of lapatinib than effects on EGFR, and the HER2 status is a determinant of lapatinib activity while EGFR status is apparently not. Pre-clinically, lapatinib induced potent growth arrest and/or apoptosis in EGFR- and HER2-dependent tumor cell lines and xenograft models, and blocked downstream MAPK and Akt activation [58]. *In vitro* studies demonstrated that the combination of lapatinib with anti-HER2 antibodies enhanced apoptosis of HER2-overexpressing breast cancer cells, and that lapatinib-mediated apoptosis was associated with downregulation of survivin [59]. Interestingly, resistance to lapatinib was recently shown to be mediated by increased signaling from the estrogen receptor (ER) in ER-positive HER2-overexpressing breast cancers, suggesting that co-targeting of ER and HER2 may be beneficial in this population [60].

Important to the issue of trastuzumab resistance, lapatinib was shown to inhibit growth of HER2-overexpressing breast cancer cells maintained long-term on trastuzumab [61]. We have observed that lapatinib induces significant apoptosis in trastuzumab-resistant cells to the same degree as in parental, trastuzumab-sensitive cells. Furthermore, lapatinib appears to have inhibitory effects on IGF-I signaling in the resistant cells, suggesting that its growth inhibitory activity may be due not only to anti-EGFR/HER2 activities but also to IGF-IR inhibition (Nahta R, Yuan LX, Yu D, Esteva FJ, submitted).

Exciting clinical data have strongly positioned lapatinib for FDA approval against HER2-overexpressing breast cancers. The phase I study EGF10004 examined heavily pretreated patients with EGFR-expressing and/or HER2-overexpressing MBC who were randomly assigned to one of five dose cohorts of lapatinib [62]. Four patients with trastuzumab-resistant MBC, two of whom were classified as having inflammatory breast cancer, had partial responses. A recent phase III trial of HER2-overexpressing MBC patients who were heavily pretreated and trastuzumab-refractory demonstrated that combination lapatinib and capecitabine resulted in a doubling of median time to progression and median progression-free survival (both 36.9 weeks) compared with capecitabine alone (median time to progression 19.7 weeks and progression-free survival 17.9 weeks) [63]. Such results are rarely if ever seen in this patient population, and support lapatinib as a promising new agent for patients who have progressed on trastuzumab-based therapy.

IGF-IR inhibition
Based on preclinical evidence suggesting a role for IGF-IR signaling in the development of trastuzumab resistance [31-33], novel IGF-IR-targeted agents have been introduced into pharmaceutical testing and are being assessed in preclinical trastuzumab-resistant models. *In vitro* studies demonstrated that inhibition of HER2 signaling using trastuzumab, and inhibition of IGF-IR signaling using a

dominant negative construct produced synergistic growth inhibition of HER2-overexpressing breast cancer cells [64]. Triple combination treatment of BT474 ER-positive HER2-overexpressing breast cancer cells or MCF7 ER-positive IGF-IR-elevated breast cancer cells with ER, HER2, and IGF-IR antagonists further augmented apoptotic effects of single agents or dual combinations [65]. In addition, our data demonstrate increased apoptosis when lapatinib and the IGF-IR monoclonal antibody alpha IR3 are combined in trastuzumab-resistant cells (Nahta R, Yuan LX, Yu D, Esteva FJ, submitted). Therapeutic strategies that target both the HER2 and IGF-I signaling pathways should be studied further for potential use in cancers that progress on trastuzumab.

PI3K inhibition
Inhibitors of pathways downstream of the HER2 receptor may combat trastuzumab resistance. Perifosine is an Akt inhibitor undergoing clinical testing in patients with solid tumors and hematological malignancies [66,67]. As most Akt inhibitors have not achieved clinical development due to excessive toxicity in preclinical models, an alternative approach to blocking PI3K/Akt signaling is the use of small molecules that inactivate the kinase mTOR, which functions downstream of Akt. Three mTOR inhibitors being tested in clinical trials for patients with breast cancer and other solid tumors are CCI-779 (temsirolimus; Wyeth-Ayerst, Madison, NJ, USA), RAD001 (everolimus; Novartis, New York, NY, USA), and AP23573 (Ariad; Cambridge, MA, USA) [68,69]. Based on the results of Nagata and colleagues [11], in which low PTEN-expressing breast tumors were found to have reduced response to trastuzumab, our group launched a clinical trial of trastuzumab in combination with the mTOR inhibitor RAD001 in patients with HER2-overexpressing MBC resistant to trastuzumab-based therapy. Additionally, drug discovery programs are focusing on developing more effective, less toxic, direct inhibitors of the Akt kinase family.

Histone deacetylase inhibitors and trastuzumab
Another class of agents called histone deacetylase inhibitors is being explored in the setting of HER2-overexpressing MBC. Preclinical work demonstrated that the histone deacetylase inhibitor hydroxamic acid analogue, LAQ824, significantly reduced HER2 levels in SKBR3 and BT474 breast cancer cells by promoting proteasome-dependent degradation and reduced transcription of HER2 [70]. These effects on HER2 were associated with induction of p27kip1 and inhibition of Akt and MAPK signaling. In addition, the combination of LAQ824 with trastuzumab induced marked apoptosis *in vitro*. Another hydroxamate-based histone deacetylase inhibitor called suberoylanilide hydroxamic acid similarly reduced HER2 protein levels [71]. It was found that suberoylanilide hydroxamic acid induced acetylation of the HER2 chaperone protein heat shock protein 90 (hsp90), reducing interaction between the proteins and promoting ubiquitination and degradation of HER2. Suberoylanilide hydroxamic acid and trastuzumab combined resulted in

synergistic induction of apoptosis in BT474 and SKBR3 breast cancer cells. These *in vitro* studies paved the way for clinical trials examining the combination of histone deacetylase inhibitors with trastuzumab.

Conclusion

The clinical problem of trastuzumab resistance is becoming increasingly important as recent studies strongly support a role for trastuzumab not only in the management of metastatic disease but also in the adjuvant setting for HER2-overexpressing breast cancers. Thus, identifying the molecular mechanisms that contribute to trastuzumab resistance is more imperative than ever. Only then can we identify novel therapeutic targets toward the goal of increasing the magnitude and duration of response to trastuzumab-based treatment.

Competing interests

FJE has been a paid consultant for Genentech, Novartis, BMS, and Sanofi-Aventis, and has received research funding from Genentech and Novartis. RN has minor stock holdings in Pfizer and a sibling with minor stock holdings in GlaxoSmithKline, the maker of lapatinib, which is discussed in this review.

Acknowledgements

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