



# Mechanisms of Trastuzumab resistance in ErbB2-driven breast cancer and newer opportunities to overcome therapy resistance

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## Abstract

The Human Epidermal Growth Factor Receptor 2 (Her2, ErbB2 or Neu) is overexpressed in about 20 – 25% of breast cancers and is causally linked to oncogenesis, providing opportunities for targeted therapy. Trastuzumab (Herceptin™, Genentech Inc, San Francisco, CA), a humanized monoclonal antibody against ErbB2, is a successful example of this concept and has vastly improved the response to treatment and overall survival in a majority of ErbB2+ breast cancer patients. However, lack of response in some patients as well as relapse during the course of therapy in others, continue to challenge researchers and clinicians alike towards a better understanding of the fundamental mechanisms of Trastuzumab action and resistance to treatment. The exact *in vivo* mechanism of action of Trastuzumab remains enigmatic, given its direct effects on the ErbB2 signaling pathway as well as indirect contributions from the immune system, by virtue of the ability of Trastuzumab to elicit Antibody-Dependent Cellular Cytotoxicity. Consequently, multiple mechanisms of resistance have been proposed. We present here a comprehensive review of our current understanding of the mechanisms, both of Trastuzumab action and clinical resistance to Trastuzumab-based therapies. We also review newer strategies (based on ErbB2 receptor biology) that are being explored to overcome resistance to Trastuzumab therapy.

**Keywords:** ErbB2 (Her2/Neu), mechanism, resistance, trastuzumab

## INTRODUCTION

ErbB2 (Her2/Neu) is a member of the ErbB family of receptor tyrosine kinases (RTKs), which includes EGFR (ErbB1, Her1), ErbB3 (Her3), and ErbB4 (Her4). ErbB2 is the preferred

dimerization partner for ErbB1, 3, and 4 following growth factor stimulation by ligands such as EGF, TGF- $\alpha$ , and amphiregulin (for EGFR) or Heregulins/Neuregulins (for ErbB3/4).<sup>[1]</sup> Oncogenic signaling by ErbB2 involves a sustained activation of a number of pathways, including the Ras-Raf-MAPK pathway, which contributes to enhanced cellular proliferation, and the PI3K-Akt pathway, which imparts cell survival among other important biological effects.

Trastuzumab (or Herceptin™; manufactured by Roche-Genentech, CA, USA), a humanized monoclonal antibody against the extracellular region of ErbB2, has served as a

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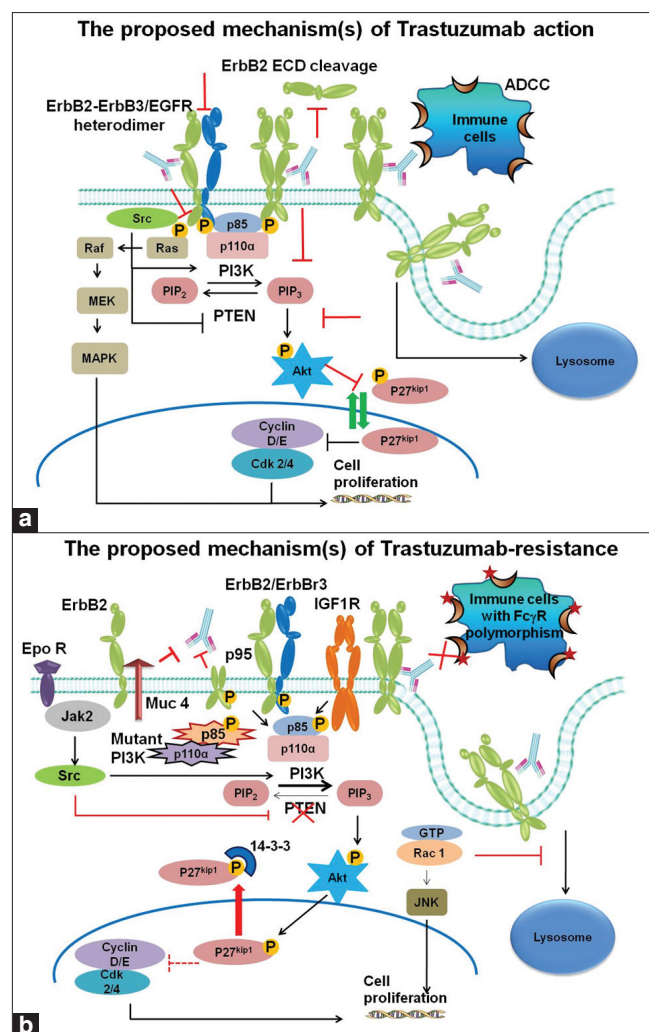
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remarkable example of a successful targeted therapeutic agent in breast cancer. Trastuzumab therapy has significantly contributed to improvements in the treatment outcome of ErbB2-driven breast cancer patients, prolonging their lives. Approximately 20 – 25% of these patients have overexpression of ErbB2, a result in most cases of the amplification of the genomic locus that includes the *ErbB2* gene. Increased levels of the ErbB2 protein can also be a result of altered transcriptional control of *ErbB2* gene expression or of biosynthetic and/or endocytic regulation of cell surface receptor levels. As the likelihood of response to Trastuzumab therapy positively correlates with the ErbB2 protein levels, patient selection typically involves assessment of the ErbB2 status by fluorescent or chromogenic *in situ* hybridization (FISH/CISH) and/or immunohistochemistry (IHC). Clinically, although monotherapy may be effective in some cases, Trastuzumab is invariably given in combination with standard chemotherapy (DNA-damaging drugs, anti-metabolites or microtubule stabilizers). Clinical studies have shown that this combination produces far better response rates than chemotherapy alone and the combinations that include Trastuzumab are now considered as the standard of care for ErbB2-overexpressing breast cancer patients.<sup>[2]</sup>

Despite the promising initial responses to Trastuzumab therapy in a majority of patients, a subset of patients fails to benefit from treatment, displaying primary or *de novo* resistance. Even within the responders, acquisition of resistance during the course of treatment (secondary resistance) is an additional challenge. Therefore, intense investigations to understand the factors that contribute to the resistance and to identify therapeutic strategies to overcome the resistance are underway at various levels, including cell biological studies, pre-clinical models, and clinical biomarker discovery. However, the effort has faced some fundamental challenges for a number of reasons. First, the exact mechanism of action of Trastuzumab, especially *in vivo*, is unclear. Second, as Trastuzumab given in combination with chemotherapy is the preferred treatment option, it has been difficult to gauge whether the clinical resistance factors are associated with the mechanism of action of Trastuzumab or that of the chemotherapeutics used in combination. Here, we make an attempt to analyze the reported findings in clinical literature, in the context of our current understanding of the mechanism of Trastuzumab as well as the potential mechanisms of its synergy, with the currently used chemotherapeutics. Furthermore, we review newer strategies based on ErbB2 receptor biology that are being explored to overcome clinical resistance associated with Trastuzumab-based therapies.

## MECHANISM OF TRASTUZUMAB ACTION

In this section, we will briefly discuss the various mechanisms of action of Trastuzumab that have been proposed, as



**Figure 1: (a) Summary of the proposed mechanism(s) of Trastuzumab action.** The therapeutic effects of Trastuzumab are thought to be mediated through its ability to activate ADCC (*in vivo*) as well as cytostasis, induced via p27<sup>kip1</sup>-mediated cell cycle arrest, which can result from its effects on the following upstream pathways – (i) ErbB2 endocytosis, ubiquitination, and degradation; (ii) blocking dimerization-dependent activation of ErbB2 with EGFR or ErbB3; (iii) inhibition of PI3K-Akt signaling; (iv) blocking ErbB2-Src interaction to reverse Src-mediated phosphorylation (inactivating) of PTEN to reactivate PTEN; (v) inhibition of metalloprotease-induced shedding of the ErbB2-extracellular domain, leading to the prevention of constitutive signaling through p95ErbB2, and (vi) immune effector-mediated cytolysis. (b) The proposed mechanism(s) that causes Trastuzumab-resistance. The potential factors that can cause resistance to Trastuzumab are: (i) PI3K hyperactivation, which can occur via multiple pathways, such as, signaling from alternative growth factor receptors (EGFR, ErbB3, and IGF-1R or p95ErbB2), PTEN-loss (through genetic or epigenetic mechanisms) or protein inactivation (through mechanisms such as oxidation, Src-dependent phosphorylation, and ubiquitin-dependent degradation), and PI3K mutations; (ii) Cyclin E dysregulation, which can occur due to overexpression of Cyclin E or downregulation of nuclear p27<sup>kip1</sup>; (iii) reduced endocytosis and lysosomal routing of ErbB2 receptors, which can be caused due to hyperactivation of c-Src non-receptor tyrosine kinase or hyperactivation of Rac1 GTPase; (iv) loss of binding of Fcγ receptors on immune effector cells to the Fc portion of Trastuzumab due to Fcγ receptor polymorphisms.

summarized in Figure 1a.

### Attenuation of ErbB2 signaling

Functioning as a monoclonal antibody, the molecular target recognized by Trastuzumab is an antigenic region on the extracellular domain of the ErbB2 receptor. Trastuzumab binding is thought to inhibit the signaling function of ErbB2, resulting in multiple possible mechanisms by which it may exert its anti-proliferative and therapeutic function *in vitro* and *in vivo*. Trastuzumab treatment of breast cancer cells *in vitro* primarily results in the inhibition of proliferation rather than cytotoxicity, which has been linked to the interruption of PI3K-AKT signaling, resulting in increased nuclear accumulation of the cell cycle inhibitor p27<sup>Kip1</sup> (as discussed later in the text under section 2.2) and subsequent inhibition of CDK2 activity.<sup>[3-5]</sup> Mechanistically, this can result from either blockage of the homo- or heterodimer formation or signal attenuation through receptor endocytosis followed by lysosomal degradation. These are indeed two of the proposed mechanisms of action of Trastuzumab.<sup>[6,7]</sup> Although *in vitro* cell biological and biochemical studies have demonstrated (and we have confirmed) that Trastuzumab treatment can result in ErbB2 internalization and degradation,<sup>[8,9]</sup> evidence of ErbB2 downregulation *in vivo* (either in pre-clinical xenograft models or clinical studies) is lacking. In a pre-clinical study using BT-474 xenografts and comparing the effect of the anti-EGFR kinase inhibitor, ZD1839 (Gefitinib or Iressa®, AstraZeneca, UK), in combination with Trastuzumab, the authors did not observe any decrease in the ErbB2 levels in tumors harvested after treatment with Trastuzumab alone or its combination with ZD1839.<sup>[10]</sup> In a clinical study reported by Mohsin *et al.*,<sup>[11]</sup> involving data on 35 patients treated with Trastuzumab monotherapy in a neoadjuvant setting, no downregulation of ErbB2 was observed.<sup>[11]</sup> Similarly, contrary to cell biological studies, the expected increase in p27<sup>Kip1</sup> following Trastuzumab treatment was also not seen in this study. Surprisingly, the authors reported clinical tumor regressions among responders, suggesting that Trastuzumab treatment probably induced apoptosis of tumor cells. As this study involved a limited cohort of patients, expanded studies are needed to verify these findings.

Trastuzumab has also been proposed to function by inhibiting the cleavage of the N-terminal extracellular domain of full length ErbB2 (p185ErbB2), which results in a shorter C-terminal fragment of ErbB2 (described as p95ErbB2 or p95Her2) that can form a hyperactive disulfide-linked homodimer, which initiates oncogenic signaling, but is resistant to Trastuzumab as it lacks the Trastuzumab-binding region.<sup>[12]</sup> In fact, a phase II clinical study<sup>[13]</sup> found lower levels of the ErbB2-extracellular

domain (ErbB2-ECD) in the serum of patients that responded, consistent with decreased oncogenic signaling, as a result of reduced generation of p95ErbB2. Notably, the truncated forms of ErbB2 can also result from alternate translation initiation sites within the ErbB2 mRNA.<sup>[14,15]</sup> Regardless of the mechanism of the generation of p95ErbB2, it lacks the binding epitope for Trastuzumab, and therefore, can be an important determinant of Trastuzumab-refractoriness.

Another proposed mechanism of the Trastuzumab-mediated attenuation of ErbB2-PI3K-Akt signaling is through its potential to reactivate the dual phosphatase, Phosphatase and tensin homolog (PTEN), by antagonizing the interaction between ErbB2 and c-Src.<sup>[16]</sup> In this model, the authors propose that the association of the c-Src non-receptor tyrosine kinase with ErbB2 leads to the phosphorylation of tyrosine residues on the membrane-binding C2 domain of PTEN, resulting in the mislocalization of PTEN and a subsequent increase in the PIP<sub>3</sub> levels, resulting in hyperactive PI3K-Akt signaling.<sup>[16]</sup> Binding of Trastuzumab is thought to disrupt the interaction of ErbB2 with c-Src, resulting in the reactivation of PTEN, allowing it to translocate to the membrane and attenuate the PIP<sub>3</sub> levels.<sup>[16]</sup> Conversely, the protein phosphatase activity of PTEN has also been proposed to dephosphorylate c-Src, to regulate its activity.<sup>[16,17]</sup> Thus, c-Src and PTEN may constitute a complex interdependent factor that ultimately determines Trastuzumab responsiveness through its downstream effect on the PI3K-Akt pathway. Indeed PTEN was subsequently identified, through high-throughput RNA interference screens, to be one of the critical factors associated with Trastuzumab-responsiveness.<sup>[18]</sup>

The eventual outcome of the attenuation of ErbB2 signaling, by one or more of the mechanism(s) of Trastuzumab action discussed earlier, is the attenuation of PI3K-Akt signaling, which leads to cytostatic effects of Trastuzumab. One of the downstream targets of Akt, is p27<sup>Kip1</sup>, which has been implicated in the mechanism of action of Trastuzumab, as discussed herewith.

### p27<sup>Kip1</sup>-induced cell cycle arrest

As discussed earlier, interruption of the ErbB2-PI3K-AKT signaling axis, either through a Trastuzumab-induced block to receptor activation or through receptor internalization and degradation, results in a G1-cell cycle arrest via stabilization of p27<sup>Kip1</sup>. Given that one of the downstream consequences of hyperactive ErbB2 signaling is a transcriptional induction of genes involved in DNA repair,<sup>[19]</sup> it can be hypothesized that Trastuzumab-mediated interruption of ErbB2 signaling can result in an attenuated repair activity following DNA-damaging chemotherapeutics or radiation when Trastuzumab

is combined with chemotherapy or radiation; in fact, *in vitro* studies appear to support this hypothesis,<sup>[20]</sup> and may explain the superior pre-clinical as well as clinical response of Trastuzumab, in combination with Cisplatin or Doxorubicin, in comparison to Trastuzumab monotherapy.<sup>[21-24]</sup> On the other hand, the combinations of Trastuzumab with Paclitaxel or Docetaxel also exhibit pharmacological synergy *in vitro*,<sup>[25,26]</sup> and have an even better clinical outcome than with DNA-damaging drugs.<sup>[27]</sup> However, the mechanism of synergy is unclear and is more difficult to explain by the effect of Trastuzumab on DNA repair, (which follows G1- or G2/M-arrest), as taxols act at the level of microtubules to cause mitotic arrest. ErbB2-overexpression has been associated with the upregulation of Survivin,<sup>[28-30]</sup> which belongs to the Inhibitors of Apoptosis (IAP) family, but is also an important component of the kinetochore complex in association with INCENP, Aurora B, and Borealin proteins.<sup>[31]</sup> It is tempting to speculate that the mechanism of synergy may involve the downstream effects of Trastuzumab-mediated disruption of ErbB2 signaling on Survivin function in relation to mitosis.

Even as the therapeutic effect of Trastuzumab may be derived from multiple mechanisms discussed earlier, a common factor appears to be the attenuation of PI3K-Akt signaling and its consequence on cell cycle regulation. Not surprisingly, the multiple mechanisms suggested to be contributory to Trastuzumab refractoriness,<sup>[7,32]</sup> as will be discussed later in the text, also seem to converge on the PI3K-Akt pathway.

### Antibody-Dependent cellular cytotoxicity

As Trastuzumab is an intact monoclonal antibody (IgG1), the Fc $\gamma$  portion of the molecule can play a significant role in its *in vivo* activity, by its ability to engage the Fc $\gamma$  receptors on immune effector cells, such as, macrophages, NK cells or cytotoxic T cells, to elicit Antibody-Dependent Cellular Cytotoxicity (ADCC).<sup>[33-36]</sup> In fact, pre-clinical studies using F(ab')<sub>2</sub> fragments of anti-ErbB2 antibodies<sup>[37]</sup> or mice deficient in Fc $\gamma$  receptor activation<sup>[38]</sup> show severely attenuated anti-tumor responses to Trastuzumab in the xenograft models. Notably, clinical studies in neo-adjuvant settings have revealed increased leukocyte infiltration within the tumor tissue following Trastuzumab treatment.<sup>[39]</sup> A recent clinical finding that Fc $\gamma$  receptor polymorphisms may be determinants of Trastuzumab response in breast cancer patients<sup>[40]</sup> supports the potential role of ADCC in Trastuzumab-based therapies. Tumor regression, reported in clinical studies,<sup>[11,41]</sup> may also perhaps be explained by ADCC-mediated cytotoxic responses, as opposed to the cytostatic effects of Trastuzumab seen *in vitro*. When combined with chemotherapy, Trastuzumab has been clearly shown to be vastly superior,<sup>[24,42]</sup> although the exact mechanisms of synergy are unclear. Taken together, this suggests that the

ADCC-independent mechanisms are equally important in the anti-tumor activity of Trastuzumab. However, while ADCC may be a predominant mechanism in Trastuzumab monotherapy, it could have a more limited role when Trastuzumab is given in combination with chemotherapy, given the cytotoxic effects of chemotherapeutics on immune cells. This complexity has not been thoroughly addressed experimentally.

## MECHANISMS OF RESISTANCE TO TRASTUZUMAB-BASED THERAPIES

Although clinical resistance to Trastuzumab-based therapies is understood as lack of response to treatment (either *de novo* or acquired during the course of treatment), the proposed mechanisms causing resistance (or refractoriness) come primarily from *in vitro* cell culture studies, in the context of Trastuzumab monotherapy. Potential tumor cell-intrinsic resistance factors include: (1) loss or inactivation of the PTEN tumor suppressor and subsequent over-activation of the PI3K pathway;<sup>[16]</sup> (2) mutant PI3K expression;<sup>[18]</sup> (3) lack of Trastuzumab binding due to expression of p95ErbB2 or steric hindrance to the Trastuzumab-binding site on ErbB2 caused by its cell-surface association with heavily glycosylated proteins such as Muc4 or CD44-hyaluronan;<sup>[15,43,44]</sup> and (4) amplification/overexpression of Cyclin E.<sup>[45]</sup> These are summarized in Figure 1b and discussed herewith.

### Hyperactivation of PI3K-Akt pathway through PTEN-loss, PI3K mutations, alternative growth factor receptor or p95ErbB2 signaling

During the course of the treatment, several genetic or environmental alterations can accumulate within the tumor or its microenvironment, such as loss/inactivation of the PTEN tumor suppressor, activating PI3K mutations, and dependence on signaling through alternative growth factor receptors including EGFR, p95ErbB2, ErbB3, Insulin-like growth factor receptor (IGF-1R), and other RTKs.<sup>[46-48]</sup> Alternately, the interaction of tumor cells with the surrounding stroma can lead to reconditioning of the tumor microenvironment, particularly changing the abundance of activating RTK ligands present within the tumor microenvironment. For example, Wang *et al.*,<sup>[49]</sup> have proposed that TGF- $\beta$ , present within the tumor microenvironment can lead to increased shedding of ligands, Heregulin, amphiregulin, and TGF- $\alpha$ , via TACE/ADAM17 relocalization to the plasma-membrane may be a contributing factor, leading to Trastuzumab resistance.<sup>[49]</sup> All these factors can contribute to acquired-resistance to therapy.

The net effect of PTEN-loss/inactivation or expression of mutant PI3K, p95ErbB2 or alternate RTKs is the



hyperactivation of the PI3K-Akt signaling pathway. The PI3K-Akt/ PTEN signaling network constitutes a major pathway in the regulation of cell proliferation, metabolism, and anti-apoptotic signal transduction.<sup>[50]</sup> Hyperactive Akt signaling affects the activity of several of its targets,<sup>[51-53]</sup> such as, the cell cycle regulator p27<sup>kip1</sup><sup>[54]</sup> the pro-apoptotic protein BAD,<sup>[55]</sup> as well as the FOXO family of transcription factors<sup>[56,57]</sup> (which regulate the transcription of pro-apoptotic effectors) by phosphorylation. The phosphorylated target proteins remain sequestered from its site of action via binding to 14-3-3 proteins,<sup>[54,55,57,58]</sup> as is the case with p27<sup>kip1</sup>, where p27<sup>kip1</sup> is unable to enter the nucleus to inhibit CDK2/CDK4 activity.<sup>[58]</sup> Similarly, sequestration of phosphorylated-FOXO transcription factors in the cytoplasm by 14-3-3 proteins prevents transcription of its target proteins, including pro-apoptotic effectors such as BNP3L.<sup>[59]</sup>

Deregulation of PI3K-Akt signaling or loss of the *PTEN* gene correlates with ErbB2+ tumor progression and maintenance as well as Trastuzumab resistance. Efficacy of Trastuzumab depends not only on the ErbB2 status of breast tumors, but also on aberrations of the genes that encode the PI3K-Akt/ PTEN pathway.<sup>[16]</sup> In particular, retrospective evaluation of formalin-fixed paraffin-embedded tissue samples, isolated from 227 patients with metastatic breast cancer, and treated with Trastuzumab, reveal a predictive role of PIK3CA activating mutations and loss of PTEN in patient responsiveness. The shorter time to progression of metastatic breast cancer in patients correlates with the ErbB2+ status and PIK3CA mutations. However, loss of PTEN results in reduced overall survival irrespective of the ErbB2 status.<sup>[60]</sup> Interestingly, the PIK3CA activating mutations are mutually exclusive with PTEN deletion, as would be anticipated, as these two proteins catalyze the same reaction in opposite directions, to regulate the PIP<sub>3</sub> levels, and thus, there would be little selective advantage from their concurrent alterations.<sup>[61]</sup> Mutations of the *PIK3CA* genes occur at frequencies of up to 40% in human breast cancers, although the mutations are not exclusively associated with ErbB2+ breast cancers.<sup>[61-64]</sup> A majority of the activating mutations of PI3K occur within exons 9 and 20 of the *PI3KCA* gene and encode the central helical domain and C-terminal kinase domain of PI3K, respectively.<sup>[61,62,65]</sup> Expression of PIK3CA, harboring single amino acid substitutions at E545K or H1047R in the immortalized breast cancer cell line MCF-10A, results in growth factor-independent proliferation and anchorage-independent growth, as a consequence of the constitutive activation of the kinase and its downstream target, Akt. Furthermore, overexpression of wild-type PIK3CA and its constitutively-active mutants, in two ErbB2 overexpressing cell lines, BT-474 and SKBR3, confer Trastuzumab resistance<sup>[18]</sup> and abrogate the cytostatic response

to Trastuzumab. These studies suggest that the activating mutations of PIK3CA may be one mechanism of *de novo* resistance to Trastuzumab and may perhaps contribute to the lower efficacy of Trastuzumab as a monotherapy. Interestingly, a recent report has suggested that Trastuzumab resistance due to PTEN inactivation can occur via Erythropoietin Receptor (EpoR)-mediated Src activation. This is a novel mechanism in a Trastuzumab chemotherapy setting, which requires further validations, as recombinant erythropoietin is used to counter erythropenia due to chemotherapy.<sup>[66]</sup>

### Escape from cell-cycle arrest

One consequence of activation of the PI3K-Akt pathway is the G1/S phase cell-cycle progression of human mammary epithelial cells through a mechanism that is partially associated with the change in localization or downregulation of p27<sup>kip1</sup>.<sup>[67]</sup> Akt-driven phosphorylation of p27 leads to its translocation from the nucleus to the cytoplasm, thereby, inhibiting its interaction with CDK2/cyclin E1.<sup>[53]</sup> The consequence of lack of p27<sup>kip1</sup>/CDK2/cyclin E1 complex formation is a release of CDK2/cyclin E1 from inhibition, which induces cell-cycle progression and cell proliferation. Human breast cancer patients that express cytoplasmic p27<sup>kip1</sup> have reduced rates of survival. In tumors from such patients, the mislocalization of p27 is associated with the activation of Akt.<sup>[53,68,69]</sup> Interestingly, Trastuzumab or an inhibitor of PI3K can redistribute p27<sup>kip1</sup> from a cytoplasmic plus nuclear to predominately nuclear distribution in BT-474 cells. However, induction of nuclear translocation of p27 by Trastuzumab does not occur in BT-474 cells lines with acquired resistance to Trastuzumab,<sup>[70]</sup> thereby indicating that the subcellular location of p27<sup>kip1</sup> is important for the cytostatic and cytotoxic properties of Trastuzumab and resistance. Secondary to p27<sup>kip1</sup> redistribution, Trastuzumab also lengthens the half-life of p27<sup>kip1</sup> by inhibiting CDK2 activation, a prerequisite for p27<sup>kip1</sup> protein degradation via the ubiquitin proteasome pathway.<sup>[70-73]</sup> Stabilization of the p27<sup>kip1</sup> protein allows for complex formation of p27<sup>kip1</sup>/CDK2-Cyclin E, resulting in a decrease in cell proliferation. Notably, reduction of the p27<sup>kip1</sup> levels by ubiquitin proteasome-dependent degradation results in the elevation of CDK2 activity in the Trastuzumab-resistant SKBR3 cells.<sup>[72]</sup> These studies suggest that ErbB2 may regulate the functions of CDK2 and Cyclin E, and that both proteins may contribute to Trastuzumab resistance. In support of these observations, the suppression of ErbB2 expression by siRNAs in SKBR3 cells results in the decreased expression of cyclin E and the activities associated with this protein.<sup>[74]</sup> Moreover, treatment of SKBR3 cells with Trastuzumab results in a decrease in the cyclin E protein levels, relocalization of p27 into the nucleus, as well as inhibition of cell-cycle progression and activation of apoptosis.<sup>[74]</sup> Furthermore, Trastuzumab-resistant, ErbB2-

**Table 1: New therapeutic approaches under evaluation to overcome Trastuzumab refractoriness in ErbB2-driven breast cancers**

Target	Agent	Resistance mechanism targeted	Phase of clinical development	Combination with Trastuzumab	Pharmaceutical source
ErbB2/ErbB3	Pertuzumab	Alternative GFR signaling	II	Yes	Genentech (San Francisco, CA)
EGFR/ErbB2	Cetuximab	Alternative GFR signaling	II	Yes	Bristol-Myers Squibb (New York, NY)
EGFR/ErbB2	Lapatinib	Alternative GFR signaling	II	Yes	GlaxoSmithKline (Research Triangle Park, NC)
IGF1R	NVP-AEW541	Alternative GFR signaling	Preclinical		Novartis (Basel, Switzerland)
PI3K inhibitor	XLI47	Downstream signaling	I-II	Yes, Paclitaxel	Exelisis (San Francisco, CA)
Akt	Perifosine	Downstream signaling	II		Keryx Biopharmaceuticals (New York, NY)
Akt	MK2206	Downstream signaling	I	Yes, Lapatinib	Merck (Whitehouse station, NJ)
mTOR	RAD001	Downstream signaling	II	Yes	Novartis (New York, NY)
mTOR	AP23573	Downstream signaling	I	Yes	Ariad (Cambridge, MA)
Aromatase inhibitor	Aromasin	Downstream signaling	III	Yes	GlaxoSmithKline (Research Triangle Park, NC)
HSP90 inhibitor	NYP-AUY922	All	I-II	Yes	Novartis (New York, NY)
HSP90 inhibitor	Alvespimycin	All	I	Yes, Paclitaxel	Bristol-Myers Squibb (New York, NY)
HSP90 inhibitor	Tanespimycin	All	II	Yes	Bristol-Myers Squibb (New York, NY)
ErbB2	Trastuzumab-DM1	All	II		Genentech (San Francisco, CA)
Bevacizumab	Angiogenesis inhibitor	Angiogenesis	II	Yes, Paclitaxel and Carboplatin	Genentech (San Francisco, CA)
ErbB2 and Fc RI	MDX-H210	Immune	II		Medarex Inc. (Annandale, NJ)
ErbB2 and Fc RIII	2B1	Immune	II		Chiron Corporation (Emeryville, CA)
ErbB2 recombinant therapeutic vaccine	Recombinant vaccine	Immune	I-II		Corixa Corporation (Seattle, WA) and GlaxoSmithKline (Research Triangle Park, NC)
DNA vaccine	AutoVac	Immune	II		Pharmexa (Horsholm Denmark)
Immunosuppression	Rapamune	Immune	II	Yes	Genentech (San Francisco, CA)
Transcriptional inhibitor	E1A	All	I-II		Targeted Genetics Corporation (Seattle, WA)
Telomerase inhibitor	GRN163L	All	I	Yes	Geron Corporation (Menlo Park, CA)

amplified BT-474 cells overexpress Cyclin E isoforms and exhibit elevated activity associated with the CDK2-Cyclin E complex. Suppression of Cyclin E expression by siRNAs in the Trastuzumab-resistant, ErbB2-amplified BT-474 cell line results in restoration of Trastuzumab sensitivity.

Overexpression of Cyclin E may also contribute to the efficacy of Trastuzumab in the clinic. Specifically, the comparison of ErbB2+ patients with or without overexpression of Cyclin E has revealed that the coexpression of Cyclin E with ErbB2 resulted in shorter time to progression and poorer overall outcome in response to Trastuzumab.<sup>[45]</sup> In addition, patients with ErbB2+ tumors that overexpress Cyclin E have a worse prognosis in comparison to those that have ErbB2+ tumors with low Cyclin E.<sup>[75]</sup> However, an evaluation of tissue samples isolated from patients with various stages of cancer has revealed that the expression of cyclin E serves as a prognostic marker,

irrespective of the ErbB2 status.<sup>[76]</sup> Thus, these correlations need to be further explored with in-depth mechanistic analyses.

### **Evasion of immune-mediated cytotoxic responses**

The robustness of an ADCC-mediated response to Trastuzumab therapy is highly dependent on the patient's immune system.<sup>[77]</sup> Resistance mechanisms, potentially due to altered immune mechanisms include: (1) polymorphisms within Fcγ receptors expressed on immune cells that can affect the affinity for Trastuzumab Fc region binding;<sup>[40]</sup> (2) increased expression of Killer Inhibitory Receptors (KIRs) on NK cells, which can suppress NK activity;<sup>[78]</sup> (3) immunosuppression through cytokines produced by tumor cells;<sup>[79]</sup> and (4) tumor-intrinsic expression of the BH3-family of anti-apoptotic proteins that can antagonize Granzyme B-Perforin-induced apoptosis by cytotoxic lymphocytes/NK cells.<sup>[80]</sup> Again, an experimental analysis of the role of these mechanisms using appropriate animal models in

the context of combined Trastuzumab plus chemotherapy regimens should further shed light on the relative roles of immune versus signaling mechanisms in therapeutic resistance.

### Breast Cancer Stem Cells

Tumor Initiating Cells (TICs) or Cancer Stem Cells (CSCs) are thought to contribute to tumor recurrence after adjuvant treatments.<sup>[81-84]</sup> As the adjuvant therapy of ErbB2-driven breast cancers typically involves a combination of chemotherapy with Trastuzumab, it is important to understand whether the resistance is mechanistically related to the action of Trastuzumab or to the chemotherapeutic being used in the context of TICs/CSCs. Clinical studies have shown that Trastuzumab plus chemotherapy combination in an adjuvant setting has a favorable impact on the relapse rates,<sup>[85,86]</sup> suggesting that such a regimen may impact TICs/CSCs. Notably, the highest levels of ErbB2 expression have been reported within the TIC sub-population of the ErbB2-overexpressing tumor cell lines.<sup>[87,88]</sup> In the xenograft models, Trastuzumab treatment has been reported to result in the elimination of TICs/CSCs. This may explain the favorable outcomes reported in the adjuvant settings. Higher ErbB2 expression in TICs / CSCs appears to be at apparent odds with the strong correlation between the levels of ErbB2 overexpression in breast cancers and the Trastuzumab response.<sup>[89]</sup> It is, however, possible that a higher level of ErbB2 in TICs/CSCs also endures maximal ErbB2 effects in these cells, and ensures that resistance to concurrent chemotherapeutic agents due to escape of TICs/CSCs is minimized. Any potential contribution of TICs to Trastuzumab refractoriness is probably a result of concomitant genetic alterations within the TICs, such as PTEN-loss, PI3K mutations, activation of the NFκB pathway or contributions from the Notch or Wnt signaling pathways, which have been reported to play a role in the maintenance of TICs.<sup>[90]</sup> To what extent Trastuzumab resistance, whether *de novo* or after therapy, might relate to altered TIC/CSC responses to Trastuzumab needs to be further explored.

### NEW THERAPEUTIC OPPORTUNITIES BASED ON THE BIOLOGY OF ERBB2

Several new therapeutic approaches based on insights into ErbB2 receptor biology and better cell biological understanding of the potential resistance mechanisms (as depicted in Figure 1b), are being pursued and are at various stages of pre-clinical or clinical development. These are briefly summarized in Table 1 and some of the more promising approaches are discussed here.

#### Blocking alternative growth factor receptor signaling

As discussed earlier, a major signaling node in Trastuzumab action appears to be the PI3K-Akt pathway. As signaling downstream of the alternative growth factor receptors

implicated in Trastuzumab resistance (such as, EGFR, ErbB3, p95ErbB2, and IGF-1R) converge on this node, several strategies are being tested to block the ability of alternative growth factor receptors to signal. One approach to achieve signal blockage, which has recently achieved considerable success, is the use of monoclonal antibodies that prevent ErbB2 heterodimerization with EGFR or ErbB3. Pertuzumab is one such antibody developed by Genentech (South San Francisco, CA, USA), which blocks the heterodimerization of ErbB2 with other ErbB receptors, especially ErbB3.<sup>[91]</sup> Pertuzumab in combination with Trastuzumab has been seen to have a higher efficacy in pre-clinical models.<sup>[92]</sup> Several studies involving Pertuzumab are currently in the Phase II/III trials.<sup>[93]</sup> Although the results are not published yet, a recent news and analysis report has published a 'Trial Watch' in the Nature Reviews in Drug Discovery (September 2011), announcing the preliminary results from a phase III clinical trial (involving 808 patients) of Pertuzumab plus Trastuzumab in combination with Docetaxel, which has claimed to significantly extend the progression-free survival of patients.<sup>[94]</sup> Alternatively, downstream signaling from alternative RTKs can be blocked using kinase inhibitors. Lapatinib (Tykerb™, Glaxo SmithKline, UK), a dual EGFR/ErbB2 inhibitor has been recently approved for clinical use in ErbB2+ breast cancer.<sup>[95]</sup> The addition of Lapatinib, in particular, has been shown to enhance the activity of Trastuzumab in both *in vitro* and *in vivo* studies.<sup>[96]</sup> Interestingly, the inhibition of ErbB2 kinase activity leads to an increase in the total ErbB2 and ErbB3 levels,<sup>[97]</sup> which could provide for the increased binding of Trastuzumab and promote anti-tumor effects, as the ErbB2 levels positively correlate with a response to Trastuzumab.<sup>[98]</sup> The Lapatinib plus Trastuzumab combination is currently in phase II clinical studies [Table 1]. In addition to Lapatinib, which is a reversible inhibitor, several irreversible kinase inhibitors are also being evaluated in cell-based pre-clinical studies as well as in clinical studies.<sup>[99]</sup> Neratinib (HKI-272) is one such agent that has been reported to be well-tolerated with significant clinical activity in phase II studies.<sup>[100]</sup> Given the potential role of the heterogeneity of tumors with multiple alternative growth factors contributing to resistance, another approach being explored is the use of multi-targeted kinase inhibitors, such as Sorafenib, in combination with Trastuzumab.<sup>[66,101]</sup>

#### Inhibition of downstream PI3K-Akt-mTOR signaling

As increased expression of alternative growth factor receptors, PTEN-loss or mutant PIK3CA expression result in hyperactive PI3K-Akt signaling, inhibitors of PI3K, Akt, and mTOR (downstream target of Akt) as single drugs or in combination with Trastuzumab and/or chemotherapy are also being explored.<sup>[102]</sup> The PI3K inhibitor XL147 (Exelisis, San Francisco, USA) is currently in phase I/II clinical evaluations,

in combination with Paclitaxel (clinicaltrials.gov). Interestingly, a recent study by Chakrabarty *et al.*,<sup>[103]</sup> has reported that PI3K inhibition leads to upregulation of multiple receptor tyrosine kinases. Such studies should help in the future design of basic research and clinical trials involving rational therapeutic combinations targeted at feedback regulatory pathways, to improve the clinical efficacy of Trastuzumab-based therapies. Targeting of Akt, which is downstream of PI3K, has also been evaluated; however, a phase-2 study did not find any significant objective responses in breast cancer patients with metastatic disease treated with the Akt inhibitor Perifosine (Keryx Biopharmaceuticals, NY, USA).<sup>[104]</sup> On the other hand, inhibition of mTOR (downstream target of Akt) using RAD001 (Novartis, NY, USA) has been shown to improve the response to Trastuzumab in the breast cancer models of PTEN-loss and provide some clinical benefit in a phase I/II study, involving a small number of patients.<sup>[105]</sup>

### Enhancing Trastuzumab efficacy by targeting HSP90

ErbB2 as well as a number of its downstream signaling proteins, including many implicated in Trastuzumab resistance, such as, ErbB2, p95ErbB2, Akt, CDK2, and cyclin E, are client proteins of the molecular chaperone HSP90. The kinase domains of both full-length ErbB2 and p95ErbB2, form complexes with HSP90 in cultured breast cancer cells and tumors.<sup>[106-108]</sup> The association of ErbB2 with HSP90 is necessary for the stability and activity of the nascent and mature ErbB2 protein.<sup>[9,106,107]</sup> Similar to ErbB2, the stability and activation of Akt are dependent on its interaction with HSP90.<sup>[109,110]</sup> In the case for Cdk2, only the folding and maturation of the protein is probably dependent on HSP90, as only the newly-synthesized protein associates with the chaperone.<sup>[111]</sup> Furthermore, an indirect, novel mechanism for HSP90 chaperoning for Cyclin E, may exist, as it does not directly bind HSP90, but is unstable in the presence of inhibitors of HSP90.<sup>[112]</sup>

Inhibition of the chaperone function of HSP90 using inhibitors of its ATPase activity such as geldanamycin and its derivative 17AAG results in the ubiquitination and subsequent degradation of client proteins including ErbB2, p95ErbB2, Akt, and cyclin E. Specifically, ErbB2 is rapidly endocytosed from the plasma membrane and/or rerouted from its recycling pathway to the lysosomes, in response to HSP90 inhibition.<sup>[9,113,114]</sup> The downregulation of ErbB2 correlates with a decrease of ErbB2 signal transduction, including the inactivation of the PI3K-Akt pathway, degradation of cell cycle proteins, inhibition of cell progression, and the induction of apoptosis.<sup>[9,115,116]</sup> Although the treatment of breast cancer cells with inhibitors of HSP90 or Trastuzumab is sufficient to induce the degradation of ErbB2, a combination of drugs is more effective than either alone, and is associated with a more profound inhibition of ErbB2 signaling.<sup>[9,115]</sup> Furthermore,

this synergy also seems adequate to overcome the resistance to Trastuzumab. For example, evaluation of the xenograft models of Trastuzumab-resistance driven by ErbB2 alone or ErbB2 and p95ErbB2 has shown that a combination of Trastuzumab and HSP90 inhibitors results in a greater decrease of tumor growth than Trastuzumab or the inhibitor alone.<sup>[117,118]</sup> Clinically, the combination of HSP90 inhibitor Tanespimycin and Trastuzumab was also shown to act synergistically on Trastuzumab-resistant tumors.<sup>[118-120]</sup> A recent phase II clinical trial of patients with metastatic breast cancer has revealed that a majority of patients experiencing disease progression on initial Trastuzumab therapy, exhibited a partial response or stabilization of the disease when treated with a combination of Trastuzumab and Tanespimycin.<sup>[119]</sup> These studies suggest that combinatorial treatment of Trastuzumab-refractory metastatic breast cancer patients with Trastuzumab and HSP90 inhibitors can considerably improve the survival of patients with ErbB2+ breast cancer.<sup>[118,119,121]</sup>

### Exploiting overexpressed ErbB2 as an address for the targeted delivery of cytotoxic drugs

Although multiple mechanisms may contribute to Trastuzumab-resistance, it does not appear to be due to loss of ErbB2 overexpression, based on most published cell-line models (as discussed in section 5.1). Therefore, one alternative strategy to overcome Trastuzumab-refractoriness is to exploit the cell-surface overexpression of ErbB2 and the high affinity with which Trastuzumab interacts with the receptor to achieve targeted delivery of cytotoxic drugs conjugated to Trastuzumab. Trastuzumab-MCC-DM1 (T-DM1; DM1 is an anti-mitotic drug based on the Vinca alkaloid Maytansine) is an example of a successful antibody-drug conjugate, based on the rationale of using ErbB2 as an address for the specific delivery of cytotoxic drugs. T-DM1 (Genentech) has demonstrated potent and ErbB2-selective anti-cancer activity in several ErbB2-overexpressing and Trastuzumab-resistant cell-line models.<sup>[122,123]</sup> T-DM1 has also recently completed phase I and II clinical studies,<sup>[124,125]</sup> and has been found to be well-tolerated with significant objective response rates and improvements in the progression-free survival of patients. This concept of ErbB2-targeted delivery of cytotoxic drugs is also being explored in conjunction with the nano-particulate drug delivery systems, which utilizes polymeric micelles or liposomes encapsulating conventional chemotherapeutics that are decorated with anti-ErbB2 antibodies, to achieve targeted delivery of the chemotherapeutic payload. These studies are currently in the early stages of development at the cell biology and pre-clinical levels.

### Targeting angiogenesis and activating immune effectors

Several additional pathways being explored as a target for



overcoming Trastuzumab refractoriness are angiogenesis inhibition using anti-VEGF-A antibody Bevacizumab (Avastin™, Genentech, San Francisco, USA), and by boosting the immune component of Trastuzumab action, using trifunctional antibodies such as Ertumaxomab (Rexomun™, Fresenius Biotech GmbH, Germany).

ErbB2-signaling has been implicated in tumor angiogenesis through the production of VEGF-A;<sup>[121,126]</sup> therefore, the inclusion of anti-VEGF-A antibody Bevacizumab has been evaluated in combination with Trastuzumab, Carboplatin, and Paclitaxel. However, the inclusion of Bevacizumab did not seem to provide significant clinical benefits.<sup>[127]</sup> In fact, in an adjuvant setting, the Bevacizumab plus Trastuzumab combination was not well-tolerated, and was casually linked to Bevacizumab-related toxicities.<sup>[127]</sup> Early studies with the trifunctional antibody Ertumaxomab (that targets ErbB2, CD3, and the activating Fcγ receptor) have shown promising activity, even in ErbB2 low-expressing cell lines.<sup>[128]</sup> The phase I clinical studies have reported that the antibody is safe and well-tolerated.<sup>[129]</sup>

## LIMITATIONS, CHALLENGES, AND FUTURE PERSPECTIVES

### Models of Trastuzumab-resistance

Despite the wealth of information, a global understanding of the mechanism of resistance to Trastuzumab-based therapies remains unclear. This is partly because of extremely limited cellular and animal models of clinical resistance available for study. Most reported studies have relied on a few cell-line models, which include: (1) BT-474 or SKBr-3 cell lines that have been selected for *in vitro* resistance by continuous culture in Trastuzumab;<sup>[70,72,118]</sup> (2) resistant clones derived from serially transplanted BT-474 xenografts in immune-compromised mice, continually treated with Trastuzumab *in vivo*;<sup>[45]</sup> (3) JIMT-1 cell line, which has been established from a patient resistant to Trastuzumab therapy.<sup>[130]</sup> The first two examples serve as the closest models for acquired resistance. However, as the tumor microenvironment, along with an intact immune system, is thought to play a major role in the cellular reprogramming that leads to resistance, the first two models may not accurately reproduce the characteristics of true acquired resistance. On the other hand, although JIMT-1, the only cell line available as a model of *de novo* resistance, has ErbB2 gene-amplification, the ErbB2 protein levels are much lower than the more-established ErbB2-overexpressing cell lines.<sup>[130,131]</sup> Moreover, its dependence on ErbB2 for growth is unclear, given the relative differences in sensitivities for growth inhibition by Lapatinib.<sup>[131]</sup> The cell line has been reported to also carry a mutant *PI3K* gene, have low expression of PTEN and also expresses high levels

of Neuregulin 1 (NRG1; a ligand for ErbB3). A systematic endeavor to establish newer cell line models representing both *de novo* as well as acquired resistance from patients as well as direct transplants of patient tumor tissue, as xenotransplants in mice, together with a more thorough characterization of transgenic models, with an intact immune system, should help increase our understanding of Trastuzumab resistance.

Newer genetically defined cell-line models can also be generated based on the identification of specific resistance factors. For example, as many studies appear to confirm PTEN loss as one of the factors, interrogation of the mechanistic role of PTEN in cell-line and experimental animal models, with stable or conditional knockdown/knockout of the *PTEN* gene will be very useful. Similarly as more information becomes available through the genomic analyses of patients who are responsive or resistant to Trastuzumab, newer models should become available. Such information could also hopefully lead to the future development of mouse models of Trastuzumab-resistance, which are currently lacking. These directions should help accelerate efforts to find solutions to overcome therapy resistance in the treatment of ErbB2-driven breast cancers.

### Identification of new factors that mediate Trastuzumab-Resistance using high-throughput screening approaches

The phosphatase and tensin homolog (PTEN) is the only factor so far identified using a high-throughput RNAi screen done on the Trastuzumab-sensitive BT-474 cell line that has a clinical correlation as a biomarker for Trastuzumab resistance. Of late, a limited siRNA library (covering human kinases and phosphatases) has been used to screen for additional Trastuzumab resistance factors.<sup>[132]</sup> The investigators identified additional factors that may be mediators of Trastuzumab resistance, including p27 phosphatase (PPM1H) and PTPN11 and three kinases (DYRK1A, STK10, and STYK1). The meager number of genes identified in the screens done to date suggests a potential limitation of loss of function strategies alone, as most Trastuzumab-resistance factors identified in other studies represent a gain of function (overexpression or mutation of genes that function as accessories or downstream components of ErbB2 signaling). Use of Trastuzumab-sensitive cell systems in loss of function approaches may therefore provide only part of the answer. Similar approaches on Trastuzumab-resistant cell lines are likely to lead to identification of a wider range of resistance factors. Conversely, the genome-wide overexpression of genes using human Open Reading Frame (ORF) libraries in Trastuzumab-sensitive cell line models could lead to the

identification of other factors. A cross-validation of hits from these independent approaches in laboratory and pre-clinical models, in conjunction with clinical assessments of the hits as the potential biomarkers of resistance, would greatly aid in identifying newer therapeutic combinations for overcoming Trastuzumab-resistance.

## CONCLUSIONS

Despite economically challenging times, years of public and private non-profit investment in basic and translational cancer research has produced a wealth of knowledge and information about the mechanisms that drive and sustain oncogenic growth in cancer cells, placing us in a good position to rapidly find solutions to treatment challenges. Specifically, our understandings of the mechanism(s) of Trastuzumab as well as pathways that contribute to resistance have significantly improved, since its approval in 1998, as reviewed here. However significant challenges continue to remain in translating these findings toward improving patient outcomes, which will require integration of the efforts of basic scientists, clinicians, and the pharmaceutical industry alike, through active collaboration. Such improvements are likely to come as future studies integrate the molecular, biochemical, and cell biological understanding of the mechanisms of Trastuzumab action and resistance, gleaned from laboratory studies, together with information from the clinical evaluation of potential resistance factors and biomarkers of the Trastuzumab response.

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