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Trastuzumab-Resistance and Breast Cancer

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1. Introduction

Evolution of therapeutic monoclonal antibodies significantly benefited from the recombinant DNA technologies that are used to generate chimeric, humanized, and human versions of monoclonal antibody to reduce the problem of immunogenicity and neutralization, as well as from understanding mechanisms of action mediated by monoclonal antibodies (Nelson et al., 2010; Reichert, 2009; Ranson & Sliwkowski , 2002). One of the significant advances in the application of monoclonal antibodies in oncology was the introduction and approval of trastuzumab, a humanized anti-HER2 antibody, for the treatment of HER2-positive breast cancer.

Despite initial successes and encouraging results, development of monoclonal antibody-based therapies faces several challenges (Yan et al., 2009). Among them are the selection of patients most likely to benefit from clinical trials and lack of understanding of mechanisms of resistance to monoclonal antibody-based therapies (Yan et al., 2009). Selection of patients most likely to benefit from clinical trials of monoclonal antibody-based therapies was initially based on the expression of the antigen targeted by the monoclonal antibody. The anti-HER-2 antibody trastuzumab was tested in patients whose breast tumors overexpress HER2 (Pegram et al., 1998) and the anti-epidermal growth factor receptor (EGFR) antibody cetuximab was used in patients with colorectal cancer and head and neck cancers that overexpress EGFR (Shin et al., 2001). Even with careful characterization of the antigen expression level in the patient population eligible for the clinical trials, primary resistance to monoclonal antibody-based therapies is a common problem. Up to 50% of EGFR-positive colorectal cancer patients are resistant to cetuximab (Saltz et al., 2004), and 74% of HER2-positive breast cancer patients are resistant to anti-HER2 antibody trastuzumab (Vogel et al., 2002).

It has emerged that the levels of antigen expression are not the only determinant of the patient response to monoclonal antibody therapies and that better understanding of the mechanisms of resistance to monoclonal antibodies in different patient subgroups has a potential to improve the effectiveness of the monoclonal antibody treatment. A retrospective analysis of the colorectal tumor samples from the patients that received cetuximab therapy indicated that EGFR-positive colorectal cancer patients with wild-type KRAS gene had increased overall

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survival, progression-free survival and improvement in the global health status compared to the patients whose tumors had KRAS mutations (Karapetis et al., 2008; Lievre et al., 2006). KRAS protein is a member of RAS super family of small GTP-binding proteins and a molecule downstream of the EGFR-mediated signaling cascade, and when aberrantly regulated, KRAS protein contributes to cancer development and progression (Karnoub & Weinberg, 2008). Cellular studies supported the role of KRAS mutations in the resistance to anti-EGFR antibody cetuximab (Benvenuti et al., 2007). Understanding of the role of the KRAS mutations in the resistance to the anti-EGFR antibody cetuximab has improved the selection of patients that are eligible for cetuximab treatment and as a consequence, cetuximab is currently approved for the treatment of EGFR-positive colorectal cancers that do not have KRAS mutations in codon 12 or 13. While KRAS mutations play a critical role in diminishing response to cetuximab in colorectal cancer patients, KRAS gene is infrequently mutated in breast cancer (Karnoub & Weinberg, 2008; Sanchez-Munoz et al., 2010), and it is therefore not likely to contribute to the resistance to anti-HER2 monoclonal antibody trastuzumab. The molecular basis for the resistance to anti-HER2 monoclonal antibody trastuzumab in breast cancer is still not well understood, and there are no clinically useful predictive biomarkers that can be used in conjunction with HER2 expression to predict the outcome of trastuzumab treatment in the HER2-positive breast cancer patients. Breast cancer is one of the most common cancers among women in the United States. It is the second leading cause of cancer death in women, after lung cancer. Women with HER2-overexpressing breast cancers have an increased risk of recurrence and shortened disease-free and overall survival rates (Press et al., 1993; Slamon et al., 1987; Slamon et al., 1989). Understanding the mechanism of resistance to trastuzumab and identifying the predictive biomarkers for the therapeutic resistance to trastuzumab could lead to important therapeutic advances.

Therapeutic monoclonal antibodies represent one of the most dynamic sectors in the biopharmaceutical industry (Reichert, 2009). Twelve monoclonal antibodies and antibodies-related products are licensed for the diagnosis and treatment of specific oncology indications in the U.S. (Table 1) (Note: gemtuzumab ozogamicin was withdrawn from the market in June 2010).

2. HER family of receptor tyrosine kinases

HER2 is a member of the HER family of receptor tyrosine kinases, which is composed of four type I receptors: EGFR/HER1/ErbB1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4. All receptors share a similar structure composed of an extracellular ligand-binding region, a single transmembrane lipophilic segment and a cytoplasmic tyrosine kinase-containing domain (Zhang et al. 2007). The extracellular ligand-binding region of HER family receptors is composed of four domains (I-IV). Domains I and III are important for ligand binding. Domain II mediates receptor dimerization. Domain IV forms intramolecular interactions with the domain II and thus blocks dimerization (Garrett et al., 2003). Ligand binding to the extracellular domain of HER family members disrupts the autoinhibition conformation which results in the receptor homo- or hetero-dimerization, and transphosphorylation followed by the activation of the downstream signaling pathways which regulate cell growth and differentiation (Hudis, 2007; Hynes & Lane, 2005). The physiological ligand for HER2 has not been identified yet. HER3 function is particularly important due to its role in the development of resistance in HER2-overexpressing cancers. HER3 binds neuregulins via its extracellular region to mediate signals primarily by heterodimerization with HER2 in

Antibody name (USAN)	Antibody tradename	Therapeutic target	Antibody type	Clinical Indication	Year of approval
Ipilimumab	Yervoy	CTLA-4	IgG1/human	Advanced melanoma	2011
Ofatumumab	Arzerra	CD20	IgG1/human	Chronic lymphocytic leukemia	2009
Panitumumab	Vectibix	EGFR	IgG2/human	Colorectal cancer	2006
Cetuximab	Erbitux	EGFR	IgG1/chimeric	Colorectal cancer Head and Neck Squamous Cell Cancer	2004 2006
Bevacizumab	Avastin	VEGF-A	IgG1/humanized	Colorectal cancer	2004
I131-Tositumomab	Bexxar	CD20	IgG2/mouse	Non-Hodgkin lymphoma	2003
Y90 or I111-Ibritumomab-tiuxetan	Zevelin	CD20	IgG1/mouse	Non-Hodgkin lymphoma	2002
Alemtuzumab	Campath	CD52	IgG1/humanized	Chronic lymphocytic leukemia	2001
Gemtuzumab Ozogamicin	Mylotarg	CD33	IgG4/humanized linked to calichaemicin	Acute myeloid leukemia	2000 withdrawn in June 2010
Trastuzumab	Herceptin	HER2	IgG1/humanized	HER2-positive breast cancer	1998
Rituximab	Rituxan	CD20	IgG1/chimeric	Non-Hodgkin lymphoma	1997
Capromab pendetide	ProstaScint	PSMA	IgG1/murine	Prostate cancer imaging	1996

Table 1. FDA approved monoclonal antibodies used for diagnosis and treatment of different oncological indications.

tumors containing amplifications of HER2 (Baselga & Swain, 2009; Shi et al., 2010). In fact, HER3-HER2 is considered the most active HER signaling dimer (Tzahar et al., 1996). HER3 also plays a key role in the ability of HER2-overexpressing cells to escape the growth inhibition by the EGFR/HER2 dual-specific tyrosine kinase inhibitor (TKI) lapatinib (Sergina et al., 2007). Previously, the intracellular kinase domain of HER3 was thought to be an inactive pseudokinase, because it lacks several key conserved and catalytically important residues (Guy et al., 1994, Sierke et al., 1997). Recently however, HER3 was shown to have

kinase activity and the ability to trans-autophosphorylate its intracellular region although it is substantially less active than EGFR (Shi et al., 2010). HER2 extracellular domain adopts a fixed conformation that resembles a ligand-activated state that permits it to form a dimer in the absence of a ligand (Cho at al., 2003; Garrett et al., 2003; Hynes & Lane, 2005). This likely explains why HER2 is the preferred dimerization partner for all of the other HER receptors (Graus-Porta et al., 1997). Moreover, although none of ligands for the HER family receptors directly binds to HER2, activation of EGFR, HER3, and HER4 can facilitate transactivation of HER2 through ligand-induced heterodimerization (Carraway et al., 1994; Wada et al., 1990).
Overexpression of HER2 has been reported in different types of cancer, including breast, gastric, ovarian and salivary gland (Baselga & Swain, 2009). Gene amplification is the most common mechanism resulting in HER2 overexpression in tumors. In addition, somatic mutations in the HER2 tyrosine kinase domain are reported in lung adenocarcinomas, epithelial ovarian cancer, hepatocellular carcinoma, gastric, colorectal and breast cancers, but the activating function for these mutations has not been clarified (Bekaii-Saab et al., 2006; Lee et al., 2006; Lin at al., 2011; Shigematsu et al., 2005). Recent studies also suggest that mutational inactivation in FOXP3 tumor suppressor may contribute to HER2 promoter activation in breast cancer tissues (Zuo et al., 2007). Regardless of the causative mechanisms resulting in HER2 overexpression in certain cancer, the number of HER2 molecules expressed on the surface of these cancer cells far exceeds the number expressed on normal cells, which facilitates the formation of HER2 heterodimers and the spontaneous formation of HER2 homodimers (Yarden & Sliwkowski, 2001). The consequence of this is an excess of HER2-mediated signaling, which drives oncogenic cell survival and proliferation (Yarden & Sliwkowski, 2001).

3. Trastuzumab and mechanisms of action of trastuzumab

For the past 20 years, the development of monoclonal antibodies and tyrosine kinase inhibitors (TKIs) targeting HER2 has been intensely pursued as important cancer therapeutic strategy. There are several reasons why HER2 is an attractive target in breast cancer treatment. First, the levels of HER2 in human cancer cells are higher than that in normal tissues and the elevated levels of HER2 correlate with the pathogenesis and prognosis in breast cancer (Natali et al., 1990; Slamon et al., 1987). Second, HER2 is overexpressed in approximately 20-30% of invasive breast cancer and is associated with poor disease-free survival and poor response to chemotherapy (Gusterson et al., 1992; Paik et al., 1990; Slamon et al., 1989). Third, HER2 is overexpressed in primary tumors and in metastatic sites suggesting that anti-HER2 therapy could be effective in all disease locations (Niehans et al., 1993). Trastuzumab is a recombinant humanized monoclonal antibody directed against the extracellular domain IV of HER2 and is approved for the treatment of HER2-positive breast cancer. In 2010, the European Medicines Agency approved trastuzumab for gastric cancer patients with high expression of HER2 (Okines et al., 2010). Subsequently, in October 2010, U.S. FDA approved trastuzumab in combination with chemotherapy for HER2-positive metastatic cancer of the stomach or the gastroesophageal junction.
Trastuzumab was engineered by inserting the antigen binding loops of a murine antibody (clone 4D5) into the framework of a consensus human IgG_1 using gene conversion mutagenesis strategy (Carter et al., 1992; Pegram et al., 1999). The humanized version of 4D5 (also known as rhuMabHER2; later named trastuzumab) showed significant effects in

HER2-overexpressing breast cancer cells and in HER2-overexpressing xenograft breast cancer models either alone or in combination with other chemotherapy agents (Pegram et al., 1999). While the mechanisms by which trastuzumab induces regression of HER2-positive breast cancers are still being investigated, it is currently believed that the binding of trastuzumab to HER2 contributes to its therapeutic effect by a) inducing HER2 endocytosis followed by receptor degradation; b) inhibiting either HER2 homodimerization or heterodimerization; c) preventing the cleavage of HER2 extracellular domain by the metalloprotease ADAM10 (Hudis, 2007). Taken together, binding of trastuzumab to the extracellular domain of HER2 reduces HER2-coupled mitogenic and pro-survival signaling pathways in tumor cells, leading to the inhibition of phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways, and the induction of the cyclin-dependent kinase inhibitor, p27 (Nahta & Esteva, 2006). Furthermore, trastuzumab is an IgG_1 subtype capable of inducing antibody-dependent cell mediated cytotoxicity (ADCC). Overexpression of HER2 in human tumor cells is closely associated with the increased angiogenesis and the expression of vascular endothelial growth factor (VEGF). Trastuzumab has also been shown to inhibit tumor angiogenesis, resulting in decreased microvessel density *in vivo* and reduced endothelial cell migration *in vitro* (Nahta & Esteva, 2006).

4. HER2-overexpression and clinical indications for trastuzumab

Trastuzumab is an anti-HER2 antibody indicated for the treatment of HER2-positive breast cancer and HER2-positive metastatic gastric or gastroesophageal (GE) junction adenocarcinoma. Three testing methodologies can be used to determine the HER2 status of the tumor samples: immunocytochemistry (IHC), fluorescence in situ hybridization (FISH), and chromogenic in situ hybridization (CISH) (Wolff et al., 2007). IHC detects the level of membrane bound HER2, whereas FISH and CISH detect the level of HER2 gene amplification. The American Society of Clinical Oncology/College of American Pathologists guideline was developed to define the status of HER2-positive breast cancer and recommended the use of a combination of IHC and FISH testing (Wolff et al., 2007). According to the guideline, a positive HER2 result is the IHC staining of 3+, and a FISH result of more than six HER2 gene copies per nucleus or a FISH ratio (HER2 gene signals to chromosome 17 signals) of more than 2.2 (Wolff et al., 2007). Trastuzumab was the first monoclonal antibody therapy to be approved with a companion diagnostic assay which was used to select the patients eligible for the trastuzumab treatment (Reichert, 2009).

Trastuzumab, when used as a single agent, produced an objective response rate up to 26% in the selected HER2-positive metastatic breast cancer patients (Vogel et al., 2002). Preclinical studies of the combinations of trastuzumab and different chemotherapy agents suggested the potential for additive and/or synergistic effects in the clinical settings (Baselga et al., 1998). Clinical studies then showed that trastuzumab in combination with chemotherapy in HER2-positive metastatic breast cancers significantly improved overall response rate, median overall survival, and time to disease progression (Burstein et al., 2001; Kaufman et al., 2009; Marty et al., 2005; O'Shaughnessy et al., 2004; Pegram et al., 1998; Perez et al., 2005; Slamon et al., 2001). Clinical benefit of trastuzumab treatment in HER2-positive metastatic breast cancer provided the rationale for testing the effects of trastuzumab treatment in the early stage HER2-positive breast cancer in the adjuvant and neoadjuvant setting. The adjuvant therapy is defined as any treatment given after the primary therapy, usually surgery, to increase the chance of long term survival, whereas neoadjuvant therapy refers to

the treatment given before the primary therapy. While many clinical trials are still ongoing, there are promising results for the use of trastuzumab treatment in the adjuvant and neoadjuvant settings. It was reported that one year of trastuzumab treatment after adjuvant chemotherapy significantly improved disease free survival and that trastuzumab combined concurrently with the chemotherapy improved the outcomes among HER2-positive breast cancer patients (Piccart-Gebhart et al., 2005). In addition to the use of trastuzumab in adjuvant settings, some of the clinical studies addressed the potential benefit of offering trastuzumab in the neoadjuvant settings. Results from the GeparQuattro study suggested that neoadjuvant combination of trastuzumab and different chemotherapy agents induced a high pathological complete response (pCR) rate with the minimal toxicities (Untch et al., 2010). pCR is defined as the complete absence of intact tumor cells in the resected specimen. Trastuzumab is now a standard of care in combination with chemotherapy for patients with HER2-positive breast cancer (Banerjee & Smith, 2010).

5. Resistance to the EGFR-targeted therapies

Targeting the EGFR has been intensely pursued in the past decade as a cancer treatment strategy. Small molecule tyrosine kinase inhibitors (TKIs) and anti-EGFR monoclonal antibodies are the primary approaches to inhibit EGFR-coupled signaling pathways. To date, three tyrosine kinase inhibitors, erlotinib, gefitinib, and lapatinib, have been approved for oncology indications. Cetuximab, a chimeric IgG_1 directed against the extracellular domain of EGFR, was originally generated from a murine antibody, 225 (Goldstein et al., 1995). Preclinical studies showed that cetuximab was more effective than the murine antibody 225 in inhibiting tumor growth in A431 human tumor xenografts model. Based on the preclinical studies, cetuximab was found to inhibit EGFR activation by preventing ligand binding, which inhibits EGFR dimerization and induces the EGFR internalization and downregulation (Goldstein et al., 1995; Mendelsohn, 2002). Cetuximab was approved in 2004 by FDA for the treatment of EGFR expressing metastatic colorectal cancer. In 2006, FDA approved the use of cetuximab for the treatment of locally advanced or regionally advanced head and neck squamous cell carcinomas (HNSCC). In 2006, panitumumab, a human antibody directed against the EGFR was also approved for the treatment of EGFR expressing metastatic colorectal cancer (Giusti et al., 2008; Hecht et al., 2009; Van Cutsem et al., 2007).

Molecular mechanisms contributing to the resistance to EGFR kinase inhibitors have been extensively studied. Important findings suggest that there is a strong relationship between the resistance to EGFR TKIs and the absence of an activating mutation in the intracellular kinase domain of the receptor. These EGFR kinase domain mutations, such as the point mutation, L858R, and in-frame deletion in exon 19 around codons 746–750, enhance the ligand-dependent activation of EGFR, and simultaneously increase the sensitivity to the TKIs (Han et al., 2005; Mitsudomi et al., 2005; Morgillo et al., 2007). It has also been demonstrated that the patients with the EGFR mutation-positive tumors have an improved response rate and survival after treatment with TKIs compared to the patients with tumors that express wild-type EGFR (Han et al., 2005; Mitsudomi et al., 2005). Therefore, the lack of these mutations can be considered a predictor of the treatment resistance to TKI (Morgillo et al, 2007). Mechanisms contributing to the primary resistance to EGFR TKIs also include genetic alterations, for example, EGFR variant III (EGFRvIII) and activating KRAS mutations. EGFRvIII lacks the ligand binding domain and is a constitutively activated mutant. It has also been reported that a point mutation in the exon 20 (T790M) in the kinase

domain of the EGFR gene occurs in tumors with acquired resistance to EGFR kinase inhibitors, gefitinib and erlotinib (Pao et al., 2005). Because mutations within HER2 have not been commonly found in HER2-overexpressing breast tumors (Zito et al., 2008), the mechanisms of resistance to EGFR kinase inhibitors may not be relevant to trastuzumab.
Although EGFR tyrosine kinase domain mutations may predict response to the TKIs, no mutations in the EGFR have been associated with resistance to the antibody-based therapies, cetuximab and panitumumab (Mukohara et al., 2005; Kruser & Wheeler, 2010). While EGFR gene amplification may be both predictive and prognostic and associated with the objective tumor response to cetuximab therapy, IHC based assay measuring EGFR expression may not be an accurate predictive factor for response to cetuximab therapy in colorectal cancer (Chung et al., 2005; Lievre et al., 2006), indicating that different mechanisms may contribute to cetuximab-resistance. One of the most reliable predictive biomarkers to emerge in the clinic has been that of the KRAS mutation status in colorectal cancer (Kruser & Wheeler, 2010). Lievre et al. reported that KRAS mutation status was predictive of resistance to cetuximab therapy. In this report, 30 metastatic colorectal carcinoma patients treated with cetuximab were analyzed for KRAS mutations. The authors reported that KRAS mutation was found in 43% (13 tumors) and was significantly associated with resistance to cetuximab therapy (Lievre et al., 2006). A larger study was performed to measure the KRAS mutation status in 113 patients with refractory metastatic colorectal cancer treated with cetuximab. The authors found that KRAS wild-type is a strong predictor of significant increase in progression-free survival (PFS) and overall survival (OS) in this cohort of patients (De Roock et al., 2008). Many other clinical trials have confirmed these findings, leading to a Provisional Clinical Opinion from the American Society for Clinical Oncology (ASCO) stating that all patients with metastatic colorectal carcinoma who are candidates for anti-EGFR antibody therapy should have their tumor tested for KRAS mutations in a Clinical Laboratory Improvement Amendments (CLIA)-accredited laboratory (Allegra et al., 2009). If codon 12 or 13 of KRAS is found to be mutated then patients with metastatic colorectal cancer should not receive anti-EGFR antibody therapy as a part of their treatment (Allegra et al., 2009).

6. Therapeutic resistance to trastuzumab

6.1 Clinical evidence for trastuzumab-resistance

While trastuzumab has significantly changed the outcome for the treatment of HER2-positive breast cancer, mechanisms contributing to trastuzumab-resistance are less well understood. Both primary and acquired resistance to trastuzumab pose a significant hurdle in the breast cancer therapy (Nahta & Esteva, 2006). Primary trastuzumab resistance refers to the lack of response to trastuzumab treatment in the patients with HER2-positive breast tumors who were never treated with trastuzumab before, whereas acquired resistance indicates that the patients who achieve initial response to trastuzumab acquire resistance to this antibody. In the study which evaluated the efficacy and the safety of trastuzumab as a single agent, the reported rate of primary trastuzumab-resistance was 74%, indicating that the vast majority of HER2-positive metastatic breast cancer patients demonstrate intrinsic resistance to single-agent trastuzumab (Vogel et al., 2002). Moreover, while addition of trastuzumab to chemotherapy in the cohort of HER2-positive metastatic breast cancer patients results in the higher response rates, and the extension of time to disease progression, the durations of response to trastuzumab in either the single-agent setting or in

the combination with chemotherapy has been reported to be 5-9 month (Kruser & Wheeler 2010; Nahta & Esteva, 2006; Slamon et al., 2001). Therefore, the majority of patients with HER2-positive breast cancer develops acquired resistance within one year. (Kruser & Wheeler 2010; Nahta & Esteva, 2006; Slamon et al., 2001). Understanding the mechanisms of trastuzumab-resistance and developing predictive biomarkers for therapeutic resistance to trastuzumab are critical to the discovery of novel agents that could overcome trastuzumab-resistance and potentially benefit HER2-positive breast cancer patients.

6.2 Preclinical studies

6.2.1 Cellular models used for the studies of trastuzumab-resistance

There are several preclinical cellular models developed to study the mechanisms of resistance to trastuzumab. HER2-positive breast cancer cell lines were chronically exposed to trastuzumab either *in vitro* (SKBR3 cell line) or in xenograft settings (BT474 cell line) in order to develop trastuzumab-resistant clones and populations (Nahta et al., 2004a; Ritter et al., 2007). In this approach, a comparison between parental trastuzumab-sensitive and derived trastuzumab-resistant clones was carried out in order to characterize the changes in the cell signaling pathways associated with trastuzumab-resistance. Recently, the JIMT-1 cell line was established from a breast cancer patient with HER2 gene amplification and primary resistance to trastuzumab and was used to study the mechanisms of resistance to trastuzumab (Tanner et al., 2004). These trastuzumab-resistant cell lines still overexpress HER2, suggesting that resistance to trastuzumab is not due to the loss of HER2 overexpression (Diermeier et al., 2005; Dokmanovic et al., 2009; Nagy et al., 2005; Ritter et al., 2007). Interestingly, it was reported recently that chronic exposure of BT-474 cells to trastuzumab gave rise to trastuzumab-resistant clones, which lost HER2 gene amplification and HER2 overexpression (Mittendorf et al., 2009). However, it is not clear whether trastuzumab eliminated HER2-overexpressing clones leaving only HER2-negative cancer clones or that the treatment with trastuzumab inhibited HER2 expression or induced downregulation of HER2, resulting in the loss of HER2 expression and resistance to trastuzumab.

6.2.2 Molecular mechanisms of trastuzumab-resistance

Trastuzumab-resistance may be broadly divided into two mechanistic categories. The first occurs at the cell membrane where aberrant regulation of HER2 results in deregulation of HER2 signaling pathways. The second results from changes to HER2-regulated intracellular signaling molecules that result in uncoupling signaling from their upstream regulation. Examples of the latter category include PTEN loss or expression of a constitutively activated PI3K mutant, PIK3CA.

Based on *in vitro* breast cancer cell models of trastuzumab-resistance, HER2 interactions with other membrane-associated proteins contribute to resistance. For example, in the JIMT-1 trastuzumab-resistant breast cancer cell line, binding of MUC4 glycoprotein to HER2 partially masks the trastuzumab binding site in HER2, resulting in reduced trastuzumab binding and contributing to trastuzumab-resistance (Nagy et al., 2005). Trastuzumab binding to HER2 in JIMT-1 cells was restored by downregulation of MUC4 protein (Nagy et al., 2005). Formation of homodimers, and heterodimers between EGFR, HER2, and HER3 due to overexpression of HER family ligands interferes with trastuzumab-mediated growth inhibition and contributes to trastuzumab-resistance (Diermeier et al., 2005; Motoyama 2002;

Ritter et al., 2007; Valabrega et al., 2005). *In vitro* models using breast cancer cells derived for resistance to trastuzumab found that Insulin-like growth factor-I receptor (IGF-IR) heterodimerizes with and phosphorylates ErbB2 suggesting that transactivation of ErbB2 by IGF1R may contribute to resistance (Nahta et al., 2005; Lu et al., 2004). Heterotrimerization of the growth factor receptors HER2, HER3 and IGF-IR in BT-474 breast cancer cells interferes with trastuzumab-associated p27 induction and therefore contributes to trastuzumab-resistance (Huang et al., 2010). Increased activity of small GTP-binding protein Rac1 interrupts with trastuzumab–induced HER2 endocytosis and degradation, resulting in the upregulated HER2-mediated signaling in SKBR3 breast cancer cells contributing to trastuzumab-resistance (Dokmanovic et al., 2009). The Met receptor tyrosine kinase, which is aberrantly expressed in breast cancer and predicts poor patient prognosis, is frequently expressed in HER2-overexpressing breast cancer cells, as well as in HER2-positive breast cancer, and Met activation protects cells against trastuzumab by abrogating p27 induction, thus contributing to trastuzumab resistance (Shattuck et al., 2008). Moreover, HER2-overexpressing breast cancer cells rapidly up-regulate Met expression after trastuzumab treatment, promoting their own resistance (Shattuck et al., 2008). As mentioned in the previous paragraph, loss of HER2 expression in HER2-overexpression breast cancer cells could be another mechanism contributing to trastuzumab resistance (Mittendorf et al., 2009). HER2 receptor initiated downstream signaling promotes cell proliferation and cell survival by the activation of RAS-MAPK and PI3K/Akt/mTOR pathways (Hudis, 2007; Zhou et al., 2004). Addition of trastuzumab to HER2-positive trastuzumab-sensitive cells results in the activation of PTEN, which acts as a tumor suppressor to induce inhibition of Akt phosphorylation and, therefore, antagonizes the PI3K/Akt survival pathway (Nagata et al., 2004). Nagata et al. reported that reducing PTEN in breast cancer cells by antisense oligonucleotides conferred trastuzumab-resistance and that inhibition of PI3K activity enhanced trastuzumab–mediated growth inhibition in a trastuzumab-resistant xenograft model (Nagata et al., 2004). PTEN knockdown in a large scale of RNA interference screening was found to be associated with selective isolation of cell clones resistant to trastuzumab (Berns et al., 2007). Constitutive activation of PI3K, such as PIK3CA mutant, can uncouple Akt signaling from the upstream regulation resulting in trastuzumab-resistance (Berns et al., 2007).

6.3 Clinical studies

Clinical studies have focused on characterization of the HER2 status and HER2-initiated downstream signaling pathways in the samples obtained from trastuzumab-sensitive patients and trastuzumab-resistant patients. A subtype of HER2-positive tumors with distinct biological and clinical features expresses a series of carboxyl terminal fragments of HER2 known as p95HER2. It is generally accepted that the p95HER2 can arise either by proteolytic shedding of the extracellular domain of the full-length HER2 by metalloproteinases ADAM10 at a site proximal to the transmembrane domain or by translation of the mRNA encoding truncated HER2 receptor from the internal initiation codons (Arribas et al., 2011). It is believed that trastuzumab has the ability to prevent proteolytic shedding of full-length HER2 on HER2-overexpressing breast cancer cells (Hudis, 2007). p95HER2 contains a hyperactive membrane anchored fragment that lacks the extracellular domain of HER2 and drives breast cancer progression *in vivo*. Of note, expression of p95HER2 fragments in transgenic mouse models leads to the generation of the

breast tumors that were more aggressive and metastatic than those driven by full-length HER2 (Pedersen et al., 2009). Expression of the p95HER2 fragment is predictive of poor prognosis and correlates with resistance to trastuzumab treatment in breast cancer patients (Arribas et al., 2011; Carney et al., 2003). This is likely due to the absence of the extracellular domain (ECD) required for trastuzumab binding (Arribas et al., 2011). The ECD of HER2 can be released into the circulation after cleavage of HER2 by the metalloproteinases ADAM10. It was reported that the prevalence of increased levels of ECD in patient serum with primary breast cancer varied between 0% and 38%, whereas in metastatic breast cancer the range was from 23% to 80% (Carney et al., 2003). Moreover, Ali et al. reported that the decrease in serum levels of HER2 ECD was positively associated with trastuzumab-sensitivity (Ali et al., 2008). In particular, in an analysis of 307 patients with metastatic breast cancer, individuals who did not achieve a significant decline (defined as ≥ 20%) in serum level of serum HER2 ECD after receiving trastuzumab had decreased benefit from trastuzumab-based therapy (Ali et al., 2008).

The analysis of *HER2* gene amplification before and after trastuzumab therapy suggested that patients who had lost *HER2* gene amplification had a significantly decreased recurrence free survival (RFS) compared with patients whose tumors remained *HER2* amplified after trastuzumab treatment (Mittendorf et al., 2009). Nagata et al. revealed that patients with PTEN-deficient breast cancers had significantly reduced responses to trastuzumab-based therapy than those with normal PTEN, suggesting PTEN deficiency may be a predictor to trastuzumab resistance (Nagata et al., 2004). Analysis of the 137 patient samples with HER2-positive metastatic breast cancer who received trastuzumab therapy revealed that activation of PI3K either by PTEN loss and/or PIK3CA mutational activation was associated with a poor response to trastuzumab and a shorter patient survival time (Esteva et al., 2010). In a neoadjuvant clinical trial, which examined the association between response to trastuzumab therapy and the status of PTEN level and PIK3CA mutations, it was found that only 15.4% of subjects with low nuclear PTEN had pathological complete response (pCR) to trastuzumab compared to 44.4% of subjects with high nuclear PTEN levels. It was reported that 20% of patients with PIK3CA activating mutations achieved pCR compared to 38.1 % of patients with wild-type PIK3 status. When the two biomarkers (PTEN level and PIK3CA status) were combined together only 18.2% of patients with low PTEN or PIK3CA achieved pCR to trastuzumab compared to 66.7 % of patients who did not have low PTEN level or PI3KAC mutations (Dave et al., 2011). Taken together, loss of PTEN and PIK3CA activating mutations are associated with trastuzumab-resistance.

Cyclin E is a critical regulator of the cell cycle G1/S transition and cyclin E levels are regulated by HER2 signaling in breast cancer cells (Mittendorf et al., 2010). Trastuzumab treatment reduces cyclin E level and activity in breast cancer cells (Mittendorf et al., 2010).

It has been recently reported that in a cohort of 34 HER2-positive breast cancer patients treated with trastuzumab-based therapy, cyclin E amplification/overexpression was associated with a worse clinical benefit and a lower progression-free survival compared with non-overexpressing cyclin E tumors, suggesting that cyclin E amplification/ overexpression may contribute to trastuzumab-resistance in HER-positive breast cancer patients (Scaltriti et al., 2011). Interestingly, in an analysis of 26 tumor samples (10 cyclin E positive and 16 cyclin E negative), cyclin E amplification only partially correlated with other clinically relevant trastuzumab-resistant markers, such as p95HER2 and PTEN loss (Scaltriti et al., 2011). This suggests that cyclin E amplification/overexpression might use a different

mechanism to contribute to trastuzumab-resistance as compared to p95HER2 and PTEN loss.

7. Toxicity and trastuzumab-related cardiotoxicity

Trastuzumab is generally well tolerated with mild to moderate side effects and low incidence of chemotherapy associated adverse events (Brufsky, 2010). The side effects reported in different clinical trials include cardiomyopathy, infusion reactions, embryo-fetal toxicity, pulmonary toxicity, exacerbation of chemotherapy-induced neutropenia, diarrhea, burning sensation in the skin, rash, and nausea and vomiting, upper respiratory tract infection, increased cough. Among those side effects, the trastuzumab-related cardiotoxicity has drawn great attention for trastuzumab-based therapy.

Trastuzumab associated cardiac dysfunction was initially reported in a phase III trial, which tested the efficacy of combining chemotherapy with trastuzumab versus chemotherapy alone in metastatic breast cancer disease (Slamon et al., 2001). The addition of trastuzumab increased the incidence of chemotherapy-associated symptomatic and asymptomatic cardiac dysfunction. These manifested as severe congestive heart failure and as significant decrease in left ventricular ejection function (LVEF) (Slamon et al., 2001; Sutter et al., 2007). In the subgroup that received anthracyclines, cyclophosphamide and trastuzumab, the incidence of cardiac dysfunction was 27% compared to 8% for the subgroup that received anthracyclines and cyclophosphamide alone (Slamon et al., 2001). For the subgroup that received paclitaxel and trastuzumab the incidence of cardiac dysfunction was 13% compared to 1% for the subgroup that received paclitaxel alone (Slamon et al., 2001). Although the results of trastuzumab adjuvant trials indicate that the incidence of trastuzumab discontinuation due to cardiac disorder was low (4.3%) and that most patients with cardiac dysfunction recovered within 6 months period (Sutter et al., 2007), the finding of trastuzumab-induced cardiac dysfunction has influenced the design of subsequent trastuzumab adjuvant trials and implementation of cardiac screening prior to and during the trastuzumab adjuvant trial (Sutter et al., 2007). Meta analysis of adjuvant trastuzumab clinical trials have assessed the incidence of cardiac dysfunction and found a favorable benefit to risk ratio for trastuzumab treatment in the early breast cancer (Viani et al., 2007).

Cancer chemotherapy, in particular anthracycline therapy, was known to be associated with cardiac dysfunction, and trastuzumab treatment increased the incidence of chemotherapy-induced cardiac dysfunction in the early trials with metastatic breast cancer patients (Slamon et al., 20001). Subsequent analysis indicated that trastuzumab-associated cardiac toxicity was clinically different than cardiac dysfunction associated with anthracyclines treatment (Ewer et al., 2005). Currently, trastuzumab-associated cardiac dysfunction is recognized as type II chemotherapy related cardiac dysfunction (Ewer & Lipmann, 2005). Clinical studies indicate that the increase in patient plasma troponin I levels, which has been proposed as an early marker of high dose chemotherapy (HDCT)-induced cardiac dysfunction, is associated with a risk of trastuzumab-induced cardiac dysfunction and the lack of left ventricular ejection fraction (LVEF) recovery (Cardinale et al., 2010). The evidence from the clinical trials indicate that trastuzumab-induced cardiomyopathy is not dose dependent, and that it is increased when trastuzumab is administered concurrently with anthracyclines, and that it is at least partially reversible (Ewer et al., 2005).

HER2 signaling in heart is essential for cardiac development and function, as well as for the prevention of dilated cardiomyopathy (Negro et al., 2004). While the mechanisms

contributing to trastuzumab-induced cardiac toxicity are still incompletely understood, it appears that blocking HER2 with anti-HER2 antibodies increases the production of reactive oxygen species and reduces human cardiomyocyte cell viability (Gordon et al., 2009). In other studies, treatment of rat cardiomyocytes with anti-HER2 antibodies increased mitochondria-dependent apoptosis by modulating the levels of Bcl-XL and Bcl-xS (Grazette et al., 2004). Consistent with the above studies, HER2 knockout mice have ventricular trabeculation deterioration, dilated cardiomyopathy, and increased sensitivity to anthracyclines toxicity (Lee et al., 1995; Negro et al., 2004). Molecular mechanisms by which trastuzumab induces cardiac dysfunction still remain elusive.

8. Strategies to overcome trastuzumab-resistance

Most examples of acquired therapeutic resistance to receptor tyrosine kinase inhibitors (TKIs) include development of mutations within the targeted receptors. For example, mutations in BCR/ABL in chronic myeloid leukemia and c-kit in gastrointestinal stromal tumors confer resistance to a specific BCR/ABL, c-kit kinase inhibitor Imatinib (Gleevec) (Litzow, 2006). Improvement of the binding of small molecules to their targets has been successfully used as a strategy to overcome resistance to imatinib (Guilhot et al.,2007; le Coutre et al.,2008).

While KRAS mutations have been found to be associated with primary resistance to cetuximab for the treatment of colorectal cancer, no predictive markers are currently used in the clinic to differentiate HER2-positive breast cancers that would respond favorably to trastuzumab from trastuzumab-resistant disease. Interestingly, KRAS, which signals downstream of both EGFR and HER2 receptors, is usually not mutated in HER2-positive breast cancer (Karnoub & Wenberg, 2008). Therefore, KRAS is not likely to contribute to therapeutic resistance to trastuzumab. Furthermore, mutations in HER2 have not been associated with resistance to trastuzumab. Based on these data, the strategies used to overcome therapeutic resistance to TKIs may not apply to trastuzumab-resistance.

8.1 Small molecule inhibitors used for the treatment of trastuzumab-resistant disease

Many new small molecule inhibitors are under clinical development to treat trastuzumab-resistant breast cancers. Early clinical studies suggest that TKIs that specifically target EGFR and HER2 have anti-tumor effects. Lapatinib, a reversible inhibitor targeting the ATP binding site of the tyrosine kinase domain of EGFR and HER2, has a mechanism of action distinct from trastuzumab. Based on the evidence obtained from preclinical and clinical studies, lapatinib activity is not dependent on the PTEN, p95HER2, and PI3K mutation status (Bartsch et al., 2007; Nahta & Esteva, 2006). Preclinical studies indicate that lapatinib is effective in inducing apoptosis in trastuzumab-resistant HER2-overexpressing breast cancer cells (Konecny et al, 2006; Nahta et al., 2007). Results from a phase I study of lapatinib in a cohort of EGFR and/or HER2 overexpressing breast cancer patients indicate that lapatinib was well tolerated and produced partial responses in patients with trastuzumab-resistant breast cancer (Burris et al., 2005). Taken together, these studies provided the rationale for clinical studies to evaluate the effect of lapatinib plus trastuzumab on duration of response. A randomized study of lapatinib alone versus the combination of lapatinib and trastuzumab in patients with trastuzumab-resistant HER2-positive metastatic breast cancer indicate that the combination of trastuzumab and lapatinib is superior to lapatinib alone for progression free survival (Blackwell et al., 2010).

Other TKIs in clinical development include neratinib which is a potent and irreversible TKI inhibitor of both EGFR and HER2. Neratinib showed promising results in clinical studies with HER2-positive breast cancer patients that were either heavily pretreated or not pretreated with trastuzumab (Burstein et al., 2010).

HER2 activates the PI3K/Akt/mammalian target of rapamycin pathway (mTOR), which represents a central signaling pathway that promotes proliferation, invasion, and survival of breast cancer cells (Zhou et al., 2004). Activation of the PI3K pathway either by loss of PTEN or by an activating mutation in PI3K, PIK3CA, is associated with lower response to anti-HER2 targeting agents, including trastuzumab (Berns et al., 2007; Nagata et al., 2004). Preclinical testing of combination of trastuzumab and PI3K, Akt or mTOR targeting agents showed that they have the potential to inhibit the growth of trastuzumab-resistant breast cancer cells and xenografts (Lu et al., 2007; Serra et al., 2008). These preclinical data are supported by recently published data of phase I trial where the oral mTOR inhibitor, everolimus, in combination with trastuzumab and vionorelbine had anti-tumor activity in HER2-positive metastatic breast cancer patients that progressed on trastuzumab (Jerusalem et al., 2011). Two additional mTOR inhibitors, rapamycin and temsirolimus, are also in clinical trials targeting trastuzumab-resistant breast cancers.

The chaperon Hsp90 has been implicated in the stabilization of a number of cellular proteins that play central roles in signaling transduction processes (Pratt & Toft, 2003). It has been reported that the intracellular domain of HER2 binds to Hsp90 and binding of Hsp90 to HER2 not only serves to maintain its physiological conformation, but also to restrain HER2 from forming active signaling dimer (Citri et al, 2004). Tanespimycin is a geldanamycin derivative that inhibits Hsp90 function in tumor cells, as well as in murine models (Zsebik et al., 2005). Cellular studies established that tanespimycin treatment either alone or in combination with trastuzumab inhibited cell growth and induced cell death in trastuzumab-sensitive and in trastuzumab-resistant cell lines (Zsebik et al., 2005). The inhibition of cell growth by tanespimycin was associated with decrease in membrane bound HER2 levels, most likely due to ubiquitination and lysosomal pathway dependent HER2 protein degradation (Raja et al., 2008). A phase I clinical testing of a combination of trastuzumab and tanespimycin showed safe and active in trastuzumab-resistant breast cancer and induced antitumor activity in HER2-positive metastatic breast cancers (Modi et al., 2007).

The following table provides information with regards to clinical trials testing multiple agents for the treatment of trastuzumab-resistant breast cancers. More detailed information can be found in the U.S. National Cancer Institute's website at http://www.cancer.gov/clinicaltrials.

8.2 Monoclonal antibodies indicated for the treatment of trastuzumab-resistant disease

Pertuzumab is an IgG_1 monoclonal antibody that binds to domain II of extracellular segment of HER2. Domain II mediates homo- and hetero-dimerization of HER2 with other members of the HER family. Therefore, binding of HER2 with pertuzumab prevents HER2-mediated dimerization and inhibits HER2-coupled signaling (Nahta et al., 2004b). Unlike pertuzumab, trastuzumab binds to domain IV of extracellular segment of HER2 receptor (Franklin et al., 2004). Cellular studies indicate that the combination of these two anti-HER2 antibodies exhibited synergistic effects in inhibiting breast cancer cell survival (Nahta et al., 2004b).

Clinical trial combination therapy for trastuzumab-resistant HER2 positive breast cancer	Molecular targets	Phase of clinical study
XL147/trastuzumab/paclitaxel	PI3K/HER2/microtubules	I,II
BKM120/trastuzumab	PI3K/HER2	I,II
AUY922/trastuzumab	Hsp90/HER2	I,II
GRN163L/trastuzumab	Telomerase/ErbB2	I
Everolimus/trastuzumab/vinorelbine	mTOR/HER2/tubulin	III
Rapamycin/trastuzumab	mTOR/HER2	II
Temsirolimus/neratinib	mTOR/EGFR, HER2	I,II
Panobinostat/trastuzumab	Histone deacetylase (HDAC)/HER2	I,II
BIBW2992/vinorelbine	EGFR, HER2/tubulin	III
BMS-754807/trastuzumab	Insulin-like growth factor-I receptor, insulin receptor/HER2	I,II

Table 2. Ongoing clinical trials testing multiple agents for trastuzumab-resistant breast cancer.

Addition of pertuzumab after progression to ongoing trastuzumab in xenografts synergistically increased tumor inhibition compared with trastuzumab alone (Friess et al., 2005). Taken together, these data suggest that trastuzumab and pertuzumab have complementary mechanisms of action and that the addition of pertuzumab to trastuzumab may improve clinical efficacy as a result of potentially broader blockade of the HER tumor cell proliferation and survival signaling (Friess et al., 2005). A Phase II trial of combination of pertuzumab and trastuzumab in HER2-positive patients that progressed on trastuzumab therapy indicated that the combination was active and well tolerated and adverse events were mild to moderate (Baselga et al., 2010). Data from this Phase II clinical trial indicated that objective response rate was 24.2%, the clinical benefit rate was 50%, and progression-free survival was 5.5 months in the cohort of patients (Baselga et al., 2010). Additionally, IMC-1121B (anti-VEGFR-2 monoclonal antibody) and IMC-18F1 (anti-VEGFR-1 monoclonal antibody) have been tested in clinical trials in combination with capecitabine, a chemotherapeutic agent that inhibits DNA synthesis and slows growth of tumor tissue, for the treatment of trastuzumab-resistant disease (National Cancer Institute, 2011; Schwartz et al., 2010; Spratin et al., 2010;).

8.3 Optimization of antibody structures

Advanced recombinant DNA technologies allow researchers to engineer therapeutic antibodies on a more rational basis. This can yield more homogeneous and stable molecules with additional properties such as increased cytotoxicity, dual-targeting, monovalent monoclonal antibodies, and enhanced penetration into solid tumors (Beck et al., 2010; Jin et al., 2008).

The variable region (Fv) of a monoclonal antibody is responsible for the binding of antibody to the antigens. Affinity maturation technology has been used to improve the binding affinity and specificity of Fv to the target. Targeting c-Met with antibodies had been difficult

because most antibodies had intrinsic agonistic activity (Prat et al., 1998). A one-armed (OA) variant of the anti-c-Met antibody 5D5 was found to act as a pure antagonist and had the ability to inhibit the growth of cells dependent on SF/HGF-c-Met autocrine and paracrine signaling (Jin et al., 2008; Nguyen et al., 2003). Data have shown that monovalent 5D5 antibody potently inhibited glioma growth in an orthotopic *in vivo* model (Martens et al., 2006).

The constant region (Fc) of an antibody is essential for the interaction between antibody and Fc receptors presenting on immune cells (Bruhns et al., 2009). Fc functions can be modulated by altering glycosylation status and binding affinity to Fc receptors, resulting in changes in antibody-dependent cellular cytotoxicity (ADCC), serum half-life, anti-inflammatory properties, and complement activation. Musolino et al. reported that the response to trastuzumab in metastatic breast cancer correlates with expression of the high affinity allele of the activating FcγRIIIa (CD16a)-158V/V (Musolino et al., 2008). It was recently reported that MGAH22, an anti-HER2 monoclonal antibody, was engineered in the Fc domain to increase binding to both alleles of the CD16a (Nordstrom et al., 2010). It was also reported that MGAH22 had enhanced activity against HER2-expressing tumors in hCD16a-158F transgenic mice (Nordstrom et al., 2010). A preclinical study showed that MGAH22 conferred enhanced activity against HER2-positive breast tumor cells, including cells resistant to trastuzumab (Nordstrom et al., 2010). Furthermore, MGAH22 exhibited greater ADCC against HER2-expressing cancer cells with lower EC_{50} (Nordstrom et al., 2010). According to the information obtained from the website of the National Cancer Institute, MGAH22 is currently in phase I clinical trials for the treatment of the patients with the HER2-positive cancers, including breast cancer, that have not responded to the standard treatment (National Cancer Institute, 2011, http://www.cancer.gov/clinicaltrials).

Multiple signaling pathways contribute to cancer development and progression. Bispecific antibodies, which are directed against two antigens that drive cancer progression, might yield better therapeutic efficacy than inhibition of a single target (Chames & Baty, 2009). Bispecific antibodies can be obtained by combining the variable domains of two already characterized monoclonal antibodies (two V_L domains on the light chain and two V_H domains on the heavy chain) using the dual variable domain IgG (DVD-IgG) technology.

This technology enables the different specificities of two monoclonal antibodies to be engineered into a single functional, dual-specific, tetravalent IgG like molecule (Beck et al., 2010). A different approach consists of engineering an additional paratope, the antigen-binding site of an antibody, in the variable domain of an existing antibody, which results in simultaneous binding to two different antigens (Beck et al., 2010; Bostrom et al., 2009). The bispecific antibody (MM-111) was developed to target both ErbB2 and ErbB3. MM-111 was indicated to displace heregulin from ErbB3 and thereby prevents receptor phosphorylation, resulting in the inhibition of tumor growth. MM-111 in combination with trastuzumab is currently in a clinical trial to treat trastuzumab-resistant breast cancer (Arnett et al., 2011; National Cancer Institute, 2011, http://www.cancer.gov/clinicaltrials).

Polyclonal or oligoclonal antibodies refer to the recombinant polycolonal or oligoclonal antibodies directed against the same or different targets. For example, the Rhesus D blood group antigen-specific polyclonal antibody rozrolimupab (Sym001; Symphogen A/S), which is a mixture of 25 unique recombinant monoclonal antibodies, is currently in Phase II clinical trials for the treatment of chronic and acute idiopathic thrombocytopenic purpura (Beck et al, 2010; Swann, et al 2008;). Hopefully, in the near future we will see more optimized monoclonal antibodies entering the clinical trials to treat trastuzumab-resistant disease.

8.4 Antibody-drug conjugates (ADC)

An important approach to delivering a lethal quantity of cytotoxic agent to the cancer cells is to select an antibody that specifically binds to a cancer-specific antigen that can mediate a rapid rate of endocytosis of antibody conjugate and accumulate them at a high concentration in cancer cells, thus resulting in cancer cell-specific killing while minimizing damage to normal cells (Chen at al., 2005; Senter, 2009). Antibody-drug conjugates are monoclonal antibody-based products that are covalently attached to the cytotoxic agent by chemical linkers (Alley et al., 2010). It is now a common strategy to develop monoclonal antibody-cytotoxic drug conjugates to improve the efficacy of both the monoclonal antibody and the cytotoxic agent for cancer indications (Chen at al, 2005; Senter, 2009). Antibody-drug conjugates consist of three different elements: the monoclonal antibody, linker, and cytotoxic agent. Three different classes of cytotoxic agents, including calicheamicin-based, maytansinoid-based, and auristatin-based cytotoxic agents, are commonly used as drugs to be conjugated to antibodies. Calicheamicin is a natural product and has been the subject of extensive research for drug delivery, due to its ability to bind to DNA in the minor groove, resulting in DNA cleavage. Maytansinoid derivatives and auristatin represent other classes of highly potent drugs that have been widely utilized for antibody-drug conjugate development. Both cytotoxic agents, maytansinoid derivatives and auristatin, act by binding to tubulin to mediate inhibition of tubulin polymerization (Chari, 2008; Doronina et al, 2006; Doronina et al. 2003). Traditionally, the antibody in an antibody-drug conjugate functions as a vehicle to carry drugs to the tumor site and drugs with high systemic toxicity are selected as payload in the antibody-drug conjugate. Gemtuzumab ozogamicin (Mylotarg), an anti-CD33 antibody conjugated to calicheamicin, was granted marketing approval for the treatment of relapsed acute myeloid leukemia in 2000. It was withdrawn from the U.S. market in June 2010 when a clinical trial showed that the drug failed to demonstrate clinical benefit to the patients enrolled in clinical trials.

Trastuzumab-DM1 (T-DM1) is an antibody-drug conjugate that was generated by linking the maytansinoid derivative maytansin to trastuzumab via a thioether linker (Lewis Phillips et al., 2008). Testing of T-DM1 in a panel of HER2-positive trastuzumab-sensitive and trastuzumab-resistant cell lines indicated that T-DM1 was cytotoxic in both trastuzumab-sensitive and trastuzumab-resistant breast cancer cell lines (Lewis Phillips et al. 2008). T-DM1 also inhibited tumor growth and caused tumor regression in trastuzumab resistant animal xenograft models (Lewis Phillips et al. 2008). T-DM1 was reported to retain the mechanisms of action of trastuzumab and was also active against lapatinib-resistant cell lines and tumors (Junttila et al., 2010). A Phase I clinical trial testing of T-DM1 reported that T-DM1 was associated with clinical activity in HER2-positive patients who had progressed on trastuzumab–based therapy (Krop et al., 2010). A Phase II study recently reported that T-DM1 had robust single-agent activity in metastatic breast cancer patients who had progressed on the previous HER2-directed therapy (Burris III et al., 2011). These clinical studies provide the evidence that the HER2 pathway remains a valid therapeutic target following disease progression on trastuzumab and suggest that antibody-drug conjugates are a novel and effective approach that can be used to treat trastuzumab-resistant disease.

8.5 Development of novel therapeutic approaches: Mechanisms of resistance-based design of antibody drug conjugates

Significant effort has been made to understand the mechanisms of resistance to trastuzumab. Many different small molecules, for example PI3 kinase inhibitors and c-Src

inhibitors have been shown to be able to revert trastuzumab-resistant phenotypes in preclinical settings (Junttila et al., 2009; Zhuang et al., 2010). Table 3 summarizes some of the novel proposed molecular targets involved in trastuzumab-resistance and small molecules that are able to override trastuzumab-resistant phenotypes based on the preclinical studies.

Molecular target implicated in trastuzumab resistance	Inhibitor for the target (preclinical studies)	Reference for the preclinical studies
Rac1	NSC23766	Dokmanovic et al., 2009
EGFR	Lapatinib Neratinib	Nahta et al.,2007 Burstein et al.,2010
PI3K	GDC-0941 LY294002 Wortmannin SF1126	Junttila et al., 2009 Clark et al., 2002 Nagata et al., 2004 Ozbay et al., 2010
Akt	Triciribine (API-2)	Lu et al.,2007
mTOR	RAD001(everolimus)	Lu et al.,2007
PI3K/mTOR	NVP-BEZ235	Serra et al., 2008
PDK-1/Akt	OSU-03012	Tseng et al., 2006
HSP90	SNX-2112 17-AAG	Chandarlapaty et al., 2010 Modi et al., 2007
TGF-β1	LY2109761	Wang et al., 2008
Src	Dasatinib	Zhuanget al., 2010
Proteasome	LLnL	Lu et al., 2004
Hyaluronan synthesis	4-MU (methylumbelliferon)	Palyi-Krekk et al., 2007
Fatty acid synthase	C-75	Vazquez-Martin et al., 2007

Table 3. Emerging molecular targets implicated in the trastuzumab-resistance and their respective inhibitors.

TGF-β is a secreted ligand that binds to type I and type II TGF-β receptors and induces the secretion of HER family ligands, such as TGF-α, amphiregulin, and heregulin. Secreted HER family ligands may enhance the association of p85 subunit of PI3K with HER3 and activate PI3K/Akt (Wang et al., 2008). Treatment with TGF-β or expression of TGF-β type I receptor in HER2-overexpressing cells reduced their sensitivity to the HER2 antibody trastuzumab. Inhibition of TGF-β type I receptor by LY2109761, a TGF-β receptor type I and type II dual inhibitor, restored sensitivity to trastuzumab (Wang et al., 2008). Rac1 is a Ras-like small GTPase which is believed to be associated with breast cancer progression and metastasis (Sahai & Marshall, 2002). Inhibition of Rac1 activity by Rac1 specific inhibitor, NSC23766, resulted in the restoration of the trastuzumab-mediated HER2 endocytic degradation and inhibition of the cell growth in trastuzumab-resistant cells (Dokmanovic et al., 2009). It has been reported that the inhibition of c-Src activity by dasatinib partially restored trastuzumab

sensitivity in trastuzumab-resistant breast cancer cells (Zhuang et al., 2010). 4-MU inhibition of hyaluronan synthase enhanced trastuzumab-mediated growth inhibition in trastuzumab-resistant JIMT-1 xenografts (Palyi-Krekk et al., 2007). Inhibition of fatty acid syntheses (FASN) re-sensitized the trastuzumab-resistant SKBR3 cells to trastuzumab-mediated cell death (Vazquez-Martin et al., 2007).

Several other approaches were utilized to interfere with the molecular pathways associated with trastuzumab-resistance in the preclinical studies. It has been recently reported that the overexpression of FoxM1, an oncogenic transcription factor, confers resistance to the trastuzumab (Carr et al., 2010). Attenuation of FoxM1 expression either by small interfering RNA or by an alternate reading frame (ARF)-derived peptide inhibitor increased the sensitivity to trastuzumab (Carr et al., 2010). Damiano et al. report that a novel toll-like receptor 9 agonist, which is also referred as the immune modulatory oligonucleotide (IMO), exerts antiangiogenic effects by cooperating with anti-EGFR or anti-VEGF antobodies, (Damiano et al., 2009). It was also shown that IMO and trastuzumab exert a cooperative antiangiogenic effect on trastuzumab-resistant breast cancer xenografts and that combining IMO and trastuzumab may be a potential strategy for the treatment of trastuzumab-resistant breast cancers (Damiano et al., 2009). The Y-box binding protein-1 (YB-1) is an oncogenic transcription/translation factor mediating expression of growth promoting genes such as EGFR and HER2. YB-1 is activated by phosphorylation at Serine 102 residue, and a decoy cell permeable peptide (CPP) functions as interference peptide to prevent endogenous YB-1 phosphorylation and activation. This results in the down-regulation of both HER-2 and EGFR transcript level and protein expression (Law et al., 2010). Interestingly, treatment with CPP has been reported to enhance sensitivity and overcome resistance to trastuzumab in cells expressing amplified HER-2, suggesting that CPP may be a novel approach for the treatment of trastuzumab-resistant breast cancers (Law et al., 2010).

Even though there were multiple mechanisms of trastuzumab-resistance proposed from the preclinical studies, the question remains how the knowledge gained from these cellular and animal models can be translated into the next generation of monoclonal antibodies to overcome therapeutic resistance to trastuzumab. Based on literature and data from our laboratory (Dokmanovic et al., 2009), we propose a new approach by designing an antibody-drug conjugate (ADC) based on mechanisms of trastuzumab resistance. In this ADC, trastuzumab is conjugated by a small molecule that has ability to inhibit the cellular target(s) that has been demonstrated to contribute to trastuzumab-resistance. This proposed strategy may increase the magnitude and duration of the response to trastuzumab treatment.

9. Conclusions

Treatment with trastuzumab significantly improves outcomes for women with HER2-positive breast cancer. However, therapeutic resistance to trastuzumab poses a significant challenge in the treatment of human breast cancer. Pre-clinical studies conducted in the past few years have improved our understanding of molecular mechanisms contributing to the trastuzumab-resistance, and potential predictive biomarkers, such as the serum levels of extracellular cellular domain (ECD) of HER2, the status of p95HER2 and IGF-IR, and loss of PTEN, have been reported. However, no predictive markers are currently used in the clinic to differentiate HER2-positive breast cancers that would respond favorably to trastuzumab from trastuzumab-resistant disease. Validation of novel predictive biomarkers must be performed with clinical samples in the context of prospective clinical trial in which

prognostic or predictive questions can be answered (Hirsch & Wu, 2007; Murphy et al., 2005). Resistance to monoclonal antibody therapeutics represents a common obstacle to the clinical efficacy for monoclonal antibody-based therapy. Understanding the molecular mechanisms of trastuzumab-resistance will lead to the discovery of new therapeutic targets, as well as more effective approaches. Innovative strategies to optimize antibody structures to develop next generation of monoclonal antibodies, such as antibody-drug conjugates, bispecific antibodies, and antibodies with either enhanced or silenced effector function, will also play a critical role in overcoming therapeutic resistance to monoclonal antibodies.

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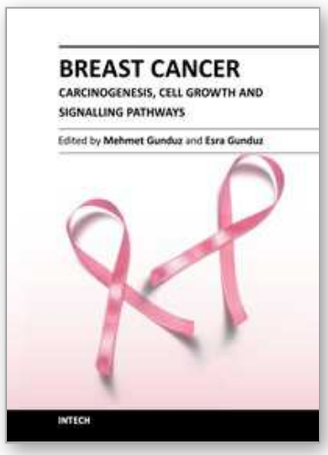

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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed various aspects of breast cancer carcinogenesis from clinics to its hormone-based as well as genetic-based etiologies for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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