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Chapter VII

Development of Trastuzumab Resistance: Is it Only in Signaling?

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Abstract

Trastuzumab (Herceptin®), a humanized monoclonal antibody to the Her-2/ErbB2 receptor, is one of the first targeted drugs to show efficacy in the treatment of breast cancer. Although patient tumors are screened for Her-2 overexpression, a significant percentage of Her-2 overexpressing tumors either fail to respond to trastuzumab (show innate resistance) or, initially respond and then become resistant during the treatment (acquired resistance). This review will address the various proposed mechanisms for developing trastuzumab resistance including cell signaling alterations and changes in the immune system that regulate antibody dependent cell killing. It is clear that tumor cell changes in cell protein expression, mutations of key cell signaling proteins, and alterations of protein compartmentalization occur in the development of *in vitro* resistance. However, surprisingly, few of these resistance strategies appear common *in vivo*. Recent findings support that alterations in the immune response may be a key mechanism in the development of *in vivo* resistance. We will discuss the development of several cell lines that express Her-2 but do not respond to trastuzumab treatment. These will be reviewed as examples of *in vitro* trastuzumab resistance and their subsequent *in vivo* predictions will be compared to observations of human resistant tumors. We will also review the attempts to correlate immune function and its suppression as a biological avenue to trastuzumab resistance. The goal of such studies should be: to determine which Her-2+ patients will have the highest likelihood of responding to trastuzumab and if there are any treatments which could reverse this resistance. For example, patients predicted to display trastuzumab resistance based on a confirmed biological assay might be good candidates to bypass trastuzumab and instead try specific tyrosine kinase inhibitors or other Her-2 specific agents as their first treatment.

Introduction

The understanding of growth factors and their receptors which lead to the development of the monoclonal antibody treatment, trastuzumab, is an exciting story and serves as a model for the development of future drugs. Here we will focus on Her-2 and the monoclonal antibody therapy, trastuzumab, however many of the ideas and concepts behind trastuzumab may also be applicable to other monoclonal antibody therapies. Her-2 expression is now measured on every breast cancer sample and patients with over-expression will most likely be given trastuzumab during some course of their treatment. Meanwhile, those patients whose tumors display low Her-2 expression will not be given trastuzumab as a treatment option due to its ineffectiveness and the increased risk for certain side effects such as cardiac toxicity. The most recent data would suggest that in the metastatic setting, the addition of trastuzumab to a chemotherapy regimen will increase the mean disease free survival by 5 to 9 months depending on the type of chemotherapy [1]. The overall survival is less pronounced where improvements are observed in only a small percent of patients. Interestingly, the use of trastuzumab alone in Her-2 + patients with metastatic disease has shown a limited response of only 25 to 30% [2-4]. However, in the area of adjuvant therapy or neoadjuvant therapy, there is a clear benefit of giving trastuzumab along with chemotherapy. When trastuzumab is added to the taxanes, a 50% reduction in disease free survival is observed [5], while patients treated in the neoadjuvant setting with chemotherapy and trastuzumab have complete responses and partial responses of 10% and 30% respectively [6].

As observed from the above response rates, the addition of trastuzumab does not result in the curing of most patients even though their tumors express Her-2. There are many Her-2+ patients that do not obtain a benefit (innate resistance) or have an initial response but then relapse while on trastuzumab (acquired resistance). This review will attempt to address the question of why these Her-2+ patients do not respond to trastuzumab based on a review of current clinical and basic research. As personalized medicine continues to gain ground, it would be beneficial to have an indicator or bioassay that could better predict which Her-2+ patients are most likely to receive benefit from trastuzumab. In the end, the oncologist would have a tool enabling them to only treat the patients that are most likely to respond to trastuzumab and not treat those who are predicted to be non-responders even though they have a Her-2+ tumor. This would save millions of dollars in health care costs (trastuzumab treatment can be as high as $100,000 per patient) and at the same time allow the patient to be treated with other agents that may be more effective in this subset of Her-2+ patients. Such Her-2+ treatment options include tyrosine kinase inhibitors [7] or the trastuzumab-chemotherapy (T-DM1) agent that works by bringing a targeted agent directly to the tumor itself [8].

A review of the literature provides many mechanisms of action for trastuzumab and the subsequent reasons for resistance that stem from tumor cell signaling and/or the lack of an effective immune response [9-12]. This chapter will briefly describe these proposed mechanisms but will spend more effort on the immunological aspects of trastuzumab since this is a major pathway that has been gaining support in recent years and may ultimately be more important than the cell signaling mechanisms. This conclusion will be substantiated by literature from our laboratory and others in this field. To the best of our knowledge, the only current assay for predicting trastuzumab treatment is the actual over-expression of Her-2. It

should also be noted that Her-2 expression can be performed by many different procedures and each has critical flaws which could affect its utility in predicting which patients should be given trastuzumab [13]. Hopefully, a newer assay that effectively predicts trastuzumab responsiveness based on a biological assay rather than a staining assay would be of benefit to the clinical community that uses monoclonal antibody therapies to treat breast and other cancers.

Non-Immunological Mechanisms for Trastuzumab Resistance

Currently there is a significant debate over what the major cell signaling mechanism of action is for trastuzumab and how resistance develops from these proposed mechanisms. Table 1 summarizes these pathways and attempts to correlate the results with what is observed clinically.

Table 1 Potential Mechanisms Via Cell Signaling for Trastuzumab Resistance and Clinical Correlations

Mechanisms of Resistance	Biological Rationale	Reason(s) Not Primary Mechanism
Rapid loss of serum trastuzumab	Lack of sufficient antibody/antigen complex	Half life of trastuzumab is 3 weeks
Low Affinity of trastuzumab to Her-2	Lack of sufficient antibody/antigen complex	Dissociation constant of trastuzumab to Her-2 is in nanomolar range
Down Regulation of Her-2	Loss of antibody/antigen complex	Not observed in patients samples
Over expression of Muc4 or CD44- hyaluronan	Prevents trastuzumab from binding to Her-2	No relationship of Muc4 or CD44 expression and patient response
Other receptors interact with Her-2 and alters signaling	trastuzuamb can not alter the signaling of these heterodimers	Few correlations of other growth factor receptor levels and response to trastuzumab
Change in expression and location of p27	Loss of cyclin dependent kinase inhibitor activity	Found in cell lines but not observed in patient samples
Changes in PTEN and mutations in PI3 kinase enzyme	Loss of aKT signaling	Clinical data mildly supports this hypothesis
Up regulation of MET receptors	Alternative signaling pathway	No relationship to clinical response
Increase in serum extracellular domain (ECD) for Her-2	Competes with trastuzumab binding	No relationship of ECD to treatment response
Loss of Trastuzumab's effect on angiogenesis, protease production, or cell migration	Cells remain more metastatic	Data not supported by clinical studies

Several of the obvious reasons for trastuzumab resistance would be expected to reside in the general pharmacokinetics of the drug. Based on this, the resistance to trastuzumab could be due to the rapid loss of the compound stemming from the metabolism of the drug or a low affinity of the drug for the target (Her-2). However, clinical studies suggest that the half life of trastuzumab is around 3 weeks and the levels of drug in the serum are sufficient to saturate the Her-2 expressing cell [14]. In addition, the affinity of trastuzumab to the Her-2 antigen is in the low nanomolar range [15]. There is data using radioactive or fluorescent trastuzumab to suggest that it does take several days for the drug to locate the tumor tissue [16]. However, this should not be the cause of the resistance since most patients are being treated for long periods of time (i.e. 6 months to 1 year). Therefore, one would not expect the above mechanisms to be major factors in the patients' resistance to trastuzumab.

Early research into the mechanism of action for trastuzumab indicated that cells treated with this drug down regulate their Her-2 expression providing an obvious reason for the development of resistance. This observation was mostly seen in cell lines [17] and was not observed in the tumors of patients that were treated with trastuzumab. In neoadjuvant studies of Her-2+ patients, the loss of Her-2 expression in tumors that remained after treatment was between 10 and 15% [14, 18]. The majority of the remaining tumors still expressed Her-2 at the same pretreatment levels. In addition, our studies indicated that Her-2 expression was not altered on cells that became resistant due to continuous exposure to trastuzumab [19]. One would therefore speculate that down regulation could happen to a particular patient but it is most likely not the major cause of trastuzumab resistance. Others have demonstrated that over-expression of a membrane glycoprotein (i.e. MUC-4) or CD44-hyaluroan complex resulted in loss of activation of Her-2 [20, 21]. A particular cell line, JIMT-1, developed from a patient resistant to trastuzumab was found to over express the MUC-4 antigen [22]. Although these observations were demonstrated in cell lines and in one patient whose tumor developed a resistance to trastuzumab, these findings have not been adequately demonstrated in other patient samples. Therefore, these studies limit the hypothesis that drug pharmacokinetics due to down regulation or blockage of receptor binding are the major causes of trastuzumab resistance.

Once trastuzumab binds to the Her-2 oncogene there is clear data, again mostly for cell lines, that cells are inhibited from growth by altering some signaling pathway and cells undergo apoptosis [9, 23-25]. The exact mechanism(s) for this inhibition/apoptosis is still controversial. However, there is clear data that suggests the importance of other growth factors receptors such as Her-1 (EGF-r), Her-3, and Her-4 playing a role in the Her-2 signaling pathway and that this interaction can be perturbed by trastuzumab binding [26, 27]. However, overexpression of these other receptors limits the effectiveness of trastuzumab. Recent data also suggested that over-expression of EphA2, a tyrosine kinase, in breast cancer cells is related to trastuzumab resistance when it was shown that EphA2 mRNA expression in patients samples related to lack of response to trastuzumab treatment [28]. This group demonstrated that inhibiting this enzyme restored trastuzumab sensitivity in both cell culture and animal studies. This is one of only a few studies that clearly demonstrate reversal of trastuzumab resistance *in vivo* with a second modulator to a specific signal protein on the tumor cells. The actual pathway by which Her-2 activates proliferation is thought to be through the Akt and/or Map Kinase pathways. Specific inhibitors of these pathways, upstream of Her-2 expression, have been shown to reverse the resistance to trastuzumab treatment and give data to suggest which pathway is most important for Her-2 biological effect [24]. Several

studies have proposed adding small molecular target inhibitors to these signaling components to enhance the biological effect of trastuzumab. In particular, tyrosine kinase inhibitors to EGF-r and to Her-2 were attempted in a clinical protocol. Unfortunately, there was no additive or synergistic effect in a randomized clinical trial [29]. Actual data to relate changes in signaling pathways to the development of trastuzumab resistance in patients are currently not available making it difficult to predict to what extent trastuzumab resistance stems from these mechanisms.

The key mechanism from a cell line perspective is that trastuzumab treated cells become growth static in nature. Our data, and that of others, have indicated that a change in cyclin dependent kinase inhibitor, p27, activity due to trastuzumab treatment is a plausible mechanism of action [19, 23, 24, 26]. These results demonstrate that signaling through the Akt pathway via Her-2 over-expression results in phosphorylation of p27 that then prevents the translocation into the nucleus. This phosphorylation step is inhibited by trastuzumab and the p27 can get into the nucleus and inhibit the cyclin dependent kinase activity resulting in cell cycle inhibition. There was also clinical data suggesting that patients with low PTEN expression [30] and/or mutations in PI3 Kinase enzymes [31] were more resistant to trastuzumab treatment. They propose the Akt pathway is the major pathway of Her-2 signaling but only demonstrate that both signals must be lost before there is any correlation to treatment response. This data has not been substantiated by other investigators which measured changes in PTEN expression in a trastuzumab neo-adjuvant trial [32]. Shattuck et al. have demonstrated development of trastuzumab resistance via up regulation of the Met receptor and this resistance is reversible by specific inhibitors. Their study again correlates inhibition through p27 as the downstream target [33]. In conclusion, there is little data beyond cell line studies to substantially support these mechanisms and even treating patients who could be refractory to trastuzumab with tyrosine kinase inhibitors still provides little benefit [29].

In other studies there were attempts to measure Her-2 levels of the extracellular domain (ECD) of Her-2 in serum and relate this to response to treatment. These levels could be related to successful killing of the tumor cells or it could mean that trastuzumab would bind to these molecules and therefore not bind to the tumor itself. However, the relationship between the ECD of Her-2 and response in a metastatic setting is limited [34]. It is also well known that interstitial fluid pressure and microvascular pressure could affect the uptake of larger molecules such as monoclonal antibodies into tumors. The characteristics of tumors that could predict this diffusion process are not really known but seem to relate to size and vascularity of the tumor. Eikenes et al. were able to demonstrate that collagenase treatment could increase the uptake of a tumor specific antibody into an osteosarcoma xenograft model [38].

In order to cover many of the other proposed mechanisms of trastuzumab resistance, one should review the role of trastuzumab on anti-angiogenesis, protease inhibition, and cell migration. The only study that extensively attempted to provide evidence in human studies was the work of Winer et al in a neoadjuvant study of trastuzumab treatment [18]. Their results demonstrated that changes in angiogenesis, in proliferation of the remaining tumor, and in protease activity did not relate to trastuzumab treatment responsiveness. However, these studies need to be repeated to confirm that trastuzumab resistant is not related to these biological features.

Immunological Mechanisms of Killing and Resistnce Development

Since trastuzumab is a monoclonal antibody and not an immunotoxin, there has been speculation that it may work mainly through the immune system rather than cell signaling pathways. In 2000, Clynes et al [35] demonstrated that expression of Fc receptors found on immune cells are necessary for effective tumor killing by trastuzumab in an animal model. His data suggested that FCγ III (-/-) animals were less sensitive to trastuzumab killing compared to FCγ III (+/+). They also demonstrated that molecular alterations of the FcγIII binding site on 4D5, the mouse monoclonal prototype for trastuzumab, resulted in loss of effective immune–induced tumor killing. The modified monoclonal antibody's loss of *in vivo* efficacy in the animal model did not correlate with a loss of the anti-proliferative effect on tumor cell lines as measured under tissue culture conditions. Several years passed before other studies confirmed these results. Gennari et al demonstrated in 2004 that there was a direct clinical correlation between antibody dependent cell mediated cytotoxicity (ADCC) and response to trastuzumab in a limited number of patients [14]. Finally, in 2006 Arnould et al found tumor responsiveness was directly correlated with an increase in the infiltration of lymphocytes, natural killer cells, and the expression of cytotoxicity agents such as granzyme B in a Her-2 + patient population neoadjuvantly treated with trastuzumab [36]. There is also data from Varchetta et al. in 2007 describing that sensitivity to trastuzumab is directly related to the number of natural killer (NK) cells which have the FCγ III receptors [37]. Based on these data it is clear that the immune system is also necessary for tumor killing using specific antibodies.

Stavenhagen et al demonstrated that they could increase the effectiveness of ADCC activity by Fc optimization. In general, those antibodies that had higher affinity via a lower dissociation rate from the Fc binding site had better ADCC. However there were exceptions to this rule [39]. A recent abstract from the 2010 ASCO meeting confirms that further modifications of trastuzumab to enhance FcγIIIa and decrease FcγIIb binding resulted in improved antibody killing in a pre-clinical setting [40]. Based on these data, one would speculate that immune competent individuals would be more likely to respond to trastuzumab treatment compared to immune suppressed individuals. However, the underlying question is how to effectively measure immune competence as it relates to trastuzumab treatment?

Our laboratory has developed an easy and reproducible assay for the measurement of ADCC killing using the xCELLigence system, a real-time cell electronic sensing system [41]. The details of the xCELLigence system will not be discussed in this review but information can be obtained on Roche's website (roche-applied-science.com) and in the following references [42-45]. With this system a higher cell index value equates to more cells in the individual well of a 96 well plate. In these studies, we demonstrate a cell line that is sensitive to trastuzumab along with a trastuzumab resistant clone of this cell line *in vitro* are both still sensitive to ADCC killing by NK cells (Figure 1) or by mononuclear cells from normal individuals (data not shown).

The advantage of this system is that one can observe the rate of killing over an extended period of time. One can quantify this killing by measuring the area under the curve for the nontreated versus the treated wells and provide specific ADCC killing activity. In a limited number of normal individuals, we observed heterogeneity of ADCC activity which was more

pronounced in the mononuclear cells compared to the polymophnuclear cells (Figure 2). Based on these observations, we would predict that patients being treated with trastuzumab would also display a similar heterogeneity.

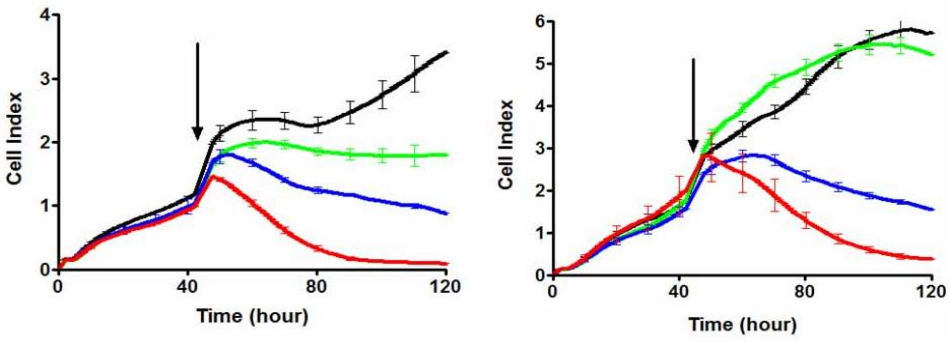

Figure 1. ADCC activity in BT474 cells (left panel) and BT474 clone #5 trastuzumab resistant cells (right panel). Cells were plated for 42 hours on the xCELLigence system and then treated with media (Black), 0.1ug/ml of trastuzumab (Green), NK92 cells (Blue) or NK92 cells + 0.1 ug/ml of trastuzuamb (Red). Area under the curve (AUC) was determined and related to ADCC activity. Used with permission of Cancer Immunol Immunother.

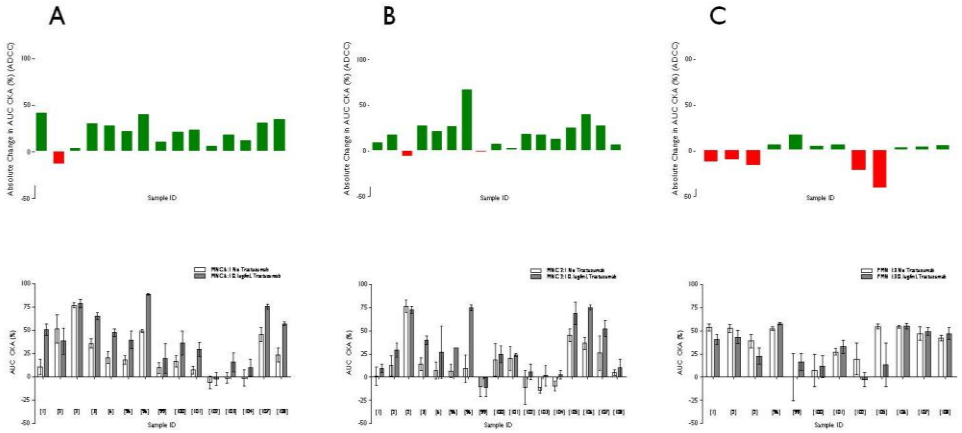

Figure 2. Measurement of ADCC activity in normal individuals using xCELLigence system. A) Mononuclear cells at an effector to target ratio of 6:1. B) Mononuclear cells at an effector to target ratio of 2:1. C) Polymorphic nuclear cells at an effector to target ratio of 1:3. Top row is the differential of killing due to trastuzumab treatment. Bottom is the raw data used for the top row calculation. Used with permission from Cancer Immunol Immunother.

There have been many different laboratories investigating the mechanism of trastuzumab resistance in breast cancer and have developed cell lines similar to ours in order to investigate this problem. Isola et al developed the cell line JIMT-1 from a patient that developed resistance to trastuzumab [46]. This cell line demonstrated several drug resistant mechanisms including an activating mutation of the PIK3CA gene, low expression of PTEN, high expression of NRG1, and relatively low expression of Her-2 receptor protein (despite testing positive for Her-2 gene amplification). Interestingly, this JIMT-1 cell line was still as

sensitive to killing by ADCC using healthy mononuclear cells as other cells lines that had over expression of Her-2 [47]. Their data also suggested that when JIMT-1 was grown in an athymic nude mouse model, it was still sensitive to trastuzumab [48]. These results further support the notion that cells deemed resistant *in vitro* could still be sensitive to trastuzumab killing *in vivo* if the patient's immune system was intact and thereby enabling ADCC killing to occur.

In another study, the Arteaga group treated a BT474 athymic nude mouse model with trastuzumab and the resistant tumors were developed into resistant cell lines [27]. They extensively characterized two cell lines and found that the resistant cells retained Her-2 expression but had an increase in the EGF-receptor expression. This over-expression resulted in higher dimerization with Her-2 and prevented trastuzumab from having its biological effect. Interesting, the wild type BT474 and the two resistant cell lines retained their ADCC activity to trastuzumab when normal human peripheral blood mononuclear cells were used as effector cells [27]. Again, these data support the fact that perturbations in cell signaling can cause resistance but do not eliminate the possibility that this resistance could be overcome by a patient with an effective immune system. Work by Nahta et al on a resistant cell line derived from SKBR-3 also support the role of increased dimerization of Her-2 with IGF-receptor which can also inhibit trastuzumab's biological effect. They went on to show that using an antibody to the IGF-1 receptor overcame the trastuzumab resistance [49]. However, it is not clear in this study whether these resistant cell lines are still sensitive to killing by a patient's immune system or by mononuclear cells

Resistance stemming from changes in the immune system will no doubt stem from a complex series of events. For example, in one study, polymorphisms of the FCγ receptors which are involved in the immune cell recognition of antigen/antibody complexes such as Her-2/trastuzumab, were shown to affect the functionality of the trastuzumab treatment. Musolina et al. demonstrated that the FCγ RIIIa-158 V/V and the FCγ IIa-131-H/H phenotypes correlated with an increase objective response rate and progression free survival [50]. They also demonstrated that these particular genotypes had higher ADCC activity compared to the other genotypes. It should be noted that others have also examined this phenotype with rituximab treatment in two studies of follicular lymphoma and in one study of Waldenstrom's macroglobulinemia [51-53]. Their results agree with Musolina's hypothesis. However a recent study in non-Hodgkin's lymphoma demonstrated no correlation of this polymorphism to the clinical outcome of rituximab treatment but did demonstrate a relationship of this polymorphism to neutropenia [54]. The same FCγ RIIIa-158 phenotype was also directly correlated to treatment of squamous cell carcinoma with cetuximab ($p<0.001$) [55]. Clearly, there is a need to better understand the interaction of the immune system with trastuzumab binding to cancer cells. It is possible that future research in this area would improve antibody-based therapies.

Potential Procedures to Overcome Trastuzumab Resistance

One potential improvement for overcoming trastuzumab resistance would be to alter the structure of the monoclonal antibody. Genentech has now been working on a chemotherapy-trastuzumab conjugate, T-DM1 [56]. The idea is that the antibody would target the chemotherapy agent, maytansinoid antimicrotubule agent, directly to the Her-2 positive cells

and provide selective killing with limited toxicity. Phase 1 and phase 2 clinical trials have demonstrated some response in patients who have been defined as refractory to trastuzumab [8]. Genentech has also developed a new humanized monoclonal antibody, pertuzamab, which functions by binding to Her-2 and inhibiting its interaction with Her-1 and Her-3 or 4. Recent results have demonstrated some effectiveness when using this compound [57].

Another potential option would be to alter the backbone of trastuzumab from an IgG to an IgE. Karagiannis et al. have demonstrated that trastuzumab with an IgE backbone works through ADCC and trastuzumab with an IgG backbone works though antibody dependent cell phagacytosis (ADCP) [58]. However, this work has yet to be replicated and no clinical trials have been used to determine which backbone would be a better antibody for selective killing of tumor cells. Kiewe et al have also proposed a trifunctional anti-Her-2 x Anti-CD3 antibody, (ertomaxomab). However, there were several reversible drug-related adverse events. The limited response rate was 33% (5 out of 15) of which one was a complete response [59]. Therefore, more work needs to be done to improve the biological effectiveness of these antibodies against cancer.

Besides changes in the antibody structure, the effectiveness of trastuzumab still relies on the functional ability of the immune system from the patient to recognize and destroy the targeted tumor cells. Therefore, other potential improvements would be to enhance the immune system prior to and/or during monoclonal antibody therapy. Even though little work has been done in this area, one would predict agents such as cytokines/interleukins or other immune-stimulating agents might improve the response to trastuzumab. However, when Rapka et al co-administered trastuzumab and IL-2 for 7 weeks they did not see any improvement in the response [60]. Meanwhile, work by others have documented that GM-CSF can stimulate rituximab immunotherapy in B-cell lymphomas [61]. There are many other agents/interventions besides these that could improve the effectiveness of trastuzumab in addition to other monoclonal antibody therapies, however a discussion of these are beyond the scope of this review.

Conclusion

There is an urgent need to understand why patients whose tumors are Her-2 positive do not respond to trastuzumab. It is clear that signaling perturbations can affect responses *in vitro*, however it unknown how influential these changes are in the *in vivo* setting. Therefore, more research is needed to better understand the immunological side of this immune-based therapy. Novel ideas have been discussed on how to overcome this resistance and need to be further studied. In addition, as treatment costs continue to rise and the era of personalized medicine continues to develop, it would be of great benefit if patients could be preselected for trastuzumab sensitivity before being exposed to the cost and risk associated with many of these drugs. In addition, physicians would be able to more effectively treat those individuals deemed unlikely to respond to standard antibody-based treatments by using other agents that would by-pass the immune-based mechanisms.

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